

To: Kelly.Petersen@LA.gov[Kelly.Petersen@LA.gov]
Cc: Palma, Ted[Palma.Ted@epa.gov]; Morris, Mark[Morris.Mark@epa.gov]
From: Strum, Madeleine
Sent: Thur 6/25/2015 1:36:31 PM
Subject: RE: priority facility emissions question

Kelly,

I checked TRI and it is way lower. Also it is important that we use actual and not permitted emissions. Could you look more into this one?

They are under 2 different TRI ids (not sure why)

1. http://oaspub.epa.gov/enviro/tris_control_v2.tris_print?tris_id=70068DPNTD560HW

			2011
CHLOROPRENE (TRI Chemical ID: 000126998)	AIR FUG	Pounds	5568
CHLOROPRENE (TRI Chemical ID: 000126998)	AIR STACK	Pounds	25470

2. http://oaspub.epa.gov/enviro/tris_control_v2.tris_print?tris_id=70069DPNTPIIHIHW

They have no reported emissions for 2011

			2013	2012	2011
CHLOROPRENE (TRI Chemical ID: 000126998)	AIR FUG	Pounds	12000	21278	NR
CHLOROPRENE (TRI Chemical ID: 000126998)	AIR STACK	Pounds	24000	228452	NR

In the 2011 NEI v2, this facility has the highest chloroprene and the facility total is higher by the 2nd highest facility by 2 orders of magnitude. The emissions really don't seem correct.

EIS Identif	Program System Code	Region	State	County	State and County FIPS	Site Name	NAICS Codes	Facility Type	Latitude	Longitude	Address	City	State	12695
802661	LADEQ08	6	LA	St. John the Baptist	22095	E I DuPont de Nemours & Co - Pontchartrain Site	325212		30.05674	90.5248	586 Hwy 44	Laplace	LA	2601
735471	LADEQ08	6	LA	East Baton Rouge	22033	Formosa Plastics Corp Louisiana	325212	Plastic, Resin, or Rubber Products Plant	30.50172	91.1858	N end of Gulf States Rd	Baton Rouge	LA	13
808471	NCDAQ	4	NC	Granville	37077	Bridgestone-Bandag, LLC	326212		36.29622	78.6104	505 West Industry Drive	Oxford	NC	338.2
625291	MSDEQ	4	MS	Tishomingo	28141	International Converter	322220		34.81488	88.1997	4309 Paul Edmondson Drive	Juka	MS	317
758671	TXCEQ	6	TX	Taylor	48441	ABILENE PLANT	326212	Plastic, Resin, or Rubber Products Plant	32.42879	99.6508	ON FM 18 W OF ELMDALE RD	ABILENE	TX	2
106256	NCDAQ	4	NC	Ashe	37009	The Gates Corporation	326212	Plastic, Resin, or Rubber Products Plant	36.4259	81.4816	401 Gates Lane	Jefferson	NC	152
136106	LADEQ08	6	LA	Iberville	22047	Shintech Louisiana LLC - Plaquemine PVC Plant	325212	Plastic, Resin, or Rubber Products Plant	30.2594	91.1737	26270 Hwy 405	Plaquemine	LA	112.6
736921	MODNR	7	MO	Greene	29077	CARLISLE POWER TRANSMISSION PRODUCTS INC- SPRINGFIELD	326220	Automobile/Truck or Parts Plant	37.16399	93.3288	2601 W BATTLEFIELD	SPRINGFIELD	MO	41.
779031	NCFCLEAD	4	NC	Forsyth	37067	HIGHLANDS INDUSTRIES	3133	Textile, Yarn, or Carpet Plant	36.12081	80.0705	215 DRUMMOND ST.	KERNERSVILLE	NC	35.22
563341	TXCEQ	6	TX	Calhoun	48057	FORMOSA POINT COMFORT PLANT	325212	Plastic, Resin, or Rubber Products Plant	28.6755	96.5493	201 FORMOSA DR	POINT COMFORT	TX	27
100021	ADEM	4	AL	Russell	1113	MeadWestvaco Mahrt Mill	3221	Pulp and Paper Plant	32.1774	85.0255	4817 Hwy 165 S	Phenix City	AL	
744211	ADEM	4	AL	Choctaw	1023	Georgia Pacific	3221	Pulp and Paper Plant	32.2272	88.0249	7530 Hwy 114	Pennington	AL	
821481	LADEQ08	6	LA	East Baton Rouge	22033	Georgia-Pacific Consumer Operations LLC - Port Hudson Operations	3221	Pulp and Paper Plant	30.65064	91.2811	4000 W Mount Pleasant Rd	Zachary	LA	19.
573401	LADEQ08	6	LA	Ouachita	22073	Graphic Packaging International Inc - West Monroe Mill #31	3221	Pulp and Paper Plant	32.4832	92.1522	4000 Jonesboro Rd	West Monroe	LA	13.
722701	LADEQ08	6	LA	Iberville	22047	Georgia Gulf Chemicals & Vinyls LLC - Plaquemine Division	325212	Plastic, Resin, or Rubber Products Plant	30.26543	91.1842	26100 Hwy 405 S	Plaquemine	LA	10.5
836111	LADEQ08	6	LA	Calcasieu	22019	Georgia Gulf Lake Charles LLC	32511		30.25228	93.2863	4600 VCM Plant Rd	Westlake	LA	8.
655961	PADEP	3	PA	Elk	42047	DOMTAR PAPER CO/JOHNSONBURG MILL	3221	Pulp and Paper Plant	41.49085	78.6775	400 W CENTER ST	JOHNSONBURG	PA	8
665051	MIDEQ	5	MI	Oakland	26125	MAC VALVES INC	332911		42.519	83.5186	30569 BECK ROAD	WIXOM	MI	7.
592941	KYDAQ	4	KY	Marshall	21157	Westlake Vinyls Inc	325998		37.0511	88.3342	2468 Industrial Pkwy	Calvert City	KY	

From: Kelly Petersen [mailto:Kelly.Petersen@LA.GOV]
Sent: Wednesday, June 24, 2015 4:01 PM
To: Strum, Madeleine
Cc: Palma, Ted
Subject: RE: priority facility emissions question

They are permitted for 170 TPY, so I think it is valid. It is also consistent with that the new owner reported this year.

Kelly Petersen

Air Permits Division

Louisiana Department of Environmental Quality

Phone: (225) 219-3397 Fax: (225) 325-8141 kelly.petersen@la.gov

From: Strum, Madeleine [mailto:Strum.Madeleine@epa.gov]
Sent: Wednesday, June 24, 2015 2:47 PM
To: Kelly Petersen
Cc: Palma, Ted
Subject: priority facility emissions question

Kelly

Can you verify the emissions of chloroprene from the below facility?

			Risk Value (cancer risk reported facility in a Emissions million) (tpy)	Facility Name	County StateNameComm
Facility ID	FIPS Tribal Parameter Code	Pollutant			
80266122095	Cancer risk	Chloroprene	16.04480.077	E I DuPont de Nemours & Co - Pontchartrain Site	St. John the LA Baptist

Thanks

Madeleine Strum
U.S. Environmental Protection Agency
Office of Air Quality Planning and Standards/Air Quality Assessment Division/EIAG
919 541 2383 (voice)
919 541 0684 (fax)

To: Rimer, Kelly[Rimer.Kelly@epa.gov]; Palma, Ted[Palma.Ted@epa.gov]; Hirtz, James[Hirtz.James@epa.gov]
From: Morris, Mark
Sent: Mon 12/14/2015 5:23:49 PM
Subject: FW: schools in laplace

The usa today link (very slow to load) indicates that it is high for cancer also, but I don't know what methodology they used to arrive at that

Mark Morris
USEPA
Mailcode C539-02
109 TW Alexander Drive
RTP, NC 27711
(919) 541-5416
morris.mark@epa.gov

From: Morris, Mark
Sent: Monday, December 14, 2015 12:20 PM
To: Rimer, Kelly <Rimer.Kelly@epa.gov>; Palma, Ted <Palma.Ted@epa.gov>; Hirtz, James <Hirtz.James@epa.gov>
Subject: schools in laplace

I downloaded the latest school data (2013) from the dept of Ed, and put them on a map. It indicates there is a school called Fifth Ward Elementary that showed up in the 1st percentile (#225) on the USA Today list (because of noncancer from chloroprene).

<http://content.usatoday.com/news/nation/environment/smokestack/school/38315>

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To: Rimer, Kelly[Rimer.Kelly@epa.gov]; Palma, Ted[Palma.Ted@epa.gov]; Hirtz, James[Hirtz.James@epa.gov]
From: Morris, Mark
Sent: Mon 12/14/2015 5:19:56 PM
Subject: schools in laplace

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<http://content.usatoday.com/news/nation/environment/smokestack/school/38315>

Mark Morris
USEPA
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109 TW Alexander Drive
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morris.mark@epa.gov

To: Doris Grego[doris-b-grego@denka-pe.com]
Cc: Palma, Ted[Palma.Ted@epa.gov]
From: Strum, Madeleine
Sent: Mon 12/14/2015 4:12:35 PM
Subject: RE: Chloroprene Concentration

Doris,

Your calculation below is correct.

Madeleine Strum
U.S. Environmental Protection Agency
Office of Air Quality Planning and Standards/Air Quality Assessment Division/EIAG
919 541 2383 (voice)
919 541 0684 (fax)

From: Doris Grego [mailto:doris-b-grego@denka-pe.com]
Sent: Monday, December 14, 2015 11:08 AM
To: Strum, Madeleine <Strum.Madeleine@epa.gov>
Subject: RE: Chloroprene Concentration

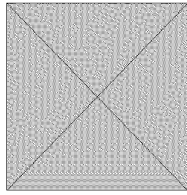
Madeleine, a quick question, based on the URE of chloroprene of 0.00048 (480E-6); an exposure concentration as low as 1.7 ug/m3 can give us a risk of 800 per million inhalation risk? I'm multiplying the concentration x the URE = $1.7 \times 480 = 816$.

Thanks for your help,

Doris B. Grego, PE

SHE Senior Consultant

985-536-5437



From: Strum, Madeleine [<mailto:Strum.Madeleine@epa.gov>]
Sent: Wednesday, July 15, 2015 11:48 AM
To: GREGO, DORIS B <Doris.B.Grego@dupont.com>
Cc: Casso, Ruben <Casso.Ruben@epa.gov>; Palma, Ted <Palma.Ted@epa.gov>; Thurman, James <Thurman.James@epa.gov>; Kelly.Petersen@LA.gov
Subject: RE: Chloroprene Concentration

Hi Doris

I have found out the below information on the unit risk estimate. Although the emissions were about the same in 2011 as they were in the 2005 NATA (confirming what you said), this issue never came up because there wasn't a URE for chloroprene at that time.

The below HEM URE (human exposure model unit risk estimate) means a chronic exposure concentration of $10.0E-6/0.00048$ or 0.02083 ug/m^3 of chloroprene over a lifetime corresponds to a 10 per million inhalation risk.

"We added the URE Chloroprene to our risk library in August 2011, we released the 2005 NATA in March 2011. The URE is based on an IRIS assessment done in 2010, the URE in IRIS is $0.0003 (1/\text{ug/m}^3)$ but this is a mutagen so we also apply a factor of 1.6 on that, thus the HEM URE is 0.00048 "

Madeleine Strum
U.S. Environmental Protection Agency
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919 541 2383 (voice)
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To: Strum, Madeleine[Strum.Madeleine@epa.gov]
Cc: Casso, Ruben[Casso.Ruben@epa.gov]; Maureen Fleming (DEQ)[M.Maureen.Fleming@LA.GOV]; Palma, Ted[Palma.Ted@epa.gov]
From: Kelly Petersen
Sent: Thur 9/17/2015 9:15:53 PM
Subject: RE: : 2011 NATA app is now available on the Sharepoint site

I do not believe we will have any comments on the NATA tract risks. As you know, I am responsible for point source data and was not a part of the air group at the time the non-point data was submitted for 2011. Beyond working to reconcile all of the high risk point source facilities with you, I am not able to verify the non-point source data or EPA calculated tract risks. It is my understanding that LDEQ used the EPA estimates along with point source subtraction for the 2011 non-point data. LDEQ does not have alternate data or a better data source to use to validate the non-point NATA data.

I will note that we have no comments on the sharepoint site as well.

Thanks,

Kelly Petersen

Air Permits Division

Louisiana Department of Environmental Quality

Phone: (225) 219-3397 Fax: (225) 325-8141 kelly.petersen@la.gov

From: Strum, Madeleine [mailto:Strum.Madeleine@epa.gov]
Sent: Tuesday, September 15, 2015 3:13 PM
To: Kelly Petersen
Cc: Casso, Ruben; Maureen Fleming (DEQ); Palma, Ted
Subject: RE: : 2011 NATA app is now available on the Sharepoint site

Kelly,

Thanks for your email and thanks for your efforts in reviewing the data and providing comments on the facility emissions. I am nearly finished updating the emissions and/or stack parameters/lons you provided. I am done with all except for Graphic Packaging which I hope to get by tomorrow. Let me know if you'd like or are able/willing to look at the update data – I've done a

lot of QA (and checked with you on some of the largest emission increases which could cause risks to go up) but another set of eyes wouldn't hurt!

I have not seen LADEQ comments on the tract risks. The tract risks incorporated modeling results from all sources of emissions (nonpoint, onroad, nonroad, fires) in addition to point. These are the results that the public will see and it is important for all states to review and provide comments. Will you be doing the review or someone else at LADEQ? Comments are due this Friday.

Thanks again,

Madeleine Strum
U.S. Environmental Protection Agency
Office of Air Quality Planning and Standards/Air Quality Assessment Division/EIAG
919 541 2383 (voice)
919 541 0684 (fax)

From: Kelly Petersen [<mailto:Kelly.Petersen@LA.GOV>]
Sent: Tuesday, September 15, 2015 3:23 PM
To: Strum, Madeleine
Cc: Casso, Ruben; Maureen Fleming (DEQ)
Subject: RE: : 2011 NATA app is now available on the Sharepoint site

Madeline,

I just wanted to check to be sure there was nothing more you needed from me with regard to the facility NATA comments. I was under the impression you were working with the facilities directly now for any additional information you need.

Thanks,

Kelly Petersen

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Phone: (225) 219-3397 Fax: (225) 325-8141 kelly.petersen@la.gov

From: Casso, Ruben [<mailto:Casso.Ruben@epa.gov>]

Sent: Tuesday, September 15, 2015 1:13 PM

To: Kelly Petersen

Subject: RE: : 2011 NATA app is now available on the Sharepoint site

Thanks Kelly. On the chloroprene draft predicted risks, I was in on a conf call between the OAQPS, the facility/consultant and I believe LDEQ. I heard OAQPS was going to remodel based on the input, but I haven't seen any new risk results yet from that additional data yet . **I should get to see a revised NATA draft once OAQPS takes all the agency comments and revises the results before going public.**

OAQPS did revise some of the risks (both up and down) in their September update, but the attached shows the highest draft predicted NATA risks I'm still seeing - as of now - in Louisiana.

From: Kelly Petersen [<mailto:Kelly.Petersen@LA.GOV>]

Sent: Tuesday, September 15, 2015 1:02 PM

To: Casso, Ruben; Jianzhong Liu

Cc: Maureen Fleming (DEQ); Amanda Polito; vennetta.hayes@la.gov

Subject: RE: : 2011 NATA app is now available on the Sharepoint site

In March, I contacted each of the point source facilities in Louisiana that were listed as high risk. Each facility was asked to submit comments and/or updated data. Those comments were

submitted to EPA via the Sharepoint site to be factored in before the preview.

I do not know of a discussion with OAQPS. If there is more I can/should do, please let me know.

Thanks,

Kelly Petersen

Air Permits Division

Louisiana Department of Environmental Quality

Phone: [\(225\) 219-3397](tel:225-219-3397) Fax: [\(225\) 325-8141](tel:225-325-8141) kelly.petersen@la.gov

From: Casso, Ruben [<mailto:Casso.Ruben@epa.gov>]

Sent: Tuesday, July 28, 2015 9:39 AM

To: Jianzhong Liu; Kelly Petersen

Subject: RE:: 2011 NATA app is now available on the Sharepoint site

Jason & Kelly – FYI:

On the NATA map apparently risks >75 in a million and >100 in a million are highlighted in darker colors. In addition to the St. John the Baptist Parish issues we discussed with OAQPS, I saw the following...

-Ruben

Total Cancer Risk is 101.43 in a million (Risks by source group and by HAP are also on a per million basis)

State LA
County St. Charles Parish
FIPS 22089062500
POP2010 2988
TotalRisk 101.43
Biogenics 14.88
Fires 3.26
Point 61.98
Nonpoint 1.30
Nonroad 2.74
Onroad 3.81
Secondary 9.67
Background 3.78

Attachments:

Risk by Broad Source Group

From: Casso, Ruben

Sent: Monday, July 27, 2015 12:42 PM

To: 'Ward, Randy'; 'FMacias@cabq.gov'; 'Davis, Anthony'; 'Jianzhong Liu'; 'Michael Honeycutt'; 'rita.bates@state.nm.us'; 'norma.perez@state.nm.us'; Kelly.Petersen@LA.gov

Subject: FW: 2011 NATA app is now available on the Sharepoint site

From: Morris, Mark

Sent: Monday, July 27, 2015 12:40 PM

To: Allen, Kara; BANDROWSKI, MIKE; Bellizzi, Carol; Brown, Steven; Cain, Alexis; campbell, dave; Casso, Ruben; Davidson, Ken; Doolan, Stephanie; Forde, Raymond; Johnson, Karen T.; King, Suzanne; Lancey, Susan; Louis, Egide; Mackintosh, David; Madrone, Brook; Narvaez, Madonna; Nwia, Jacqueline; Olson, Kyle; Palma, Ted; Parker-Christensen, Victoria; Smuts, MaryBeth; Stewart, Kathleen; Strum, Madeleine; Valdez, Heather; VanOsten, Cathleen; Wroble, Julie

Cc: Strum, Madeleine; Palma, Ted

Subject: 2011 NATA app is now available on the Sharepoint site

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Ex. 6 - Personal Privacy

Please forward this information on to your States.

Thanks.

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Cc: Casso, Ruben[Casso.Ruben@epa.gov]; Maureen Fleming (DEQ)[M.Maureen.Fleming@LA.GOV]; Palma, Ted[Palma.Ted@epa.gov]
From: Strum, Madeleine
Sent: Tue 9/15/2015 8:12:36 PM
Subject: RE: : 2011 NATA app is now available on the Sharepoint site

Kelly,

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919 541 0684 (fax)

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Cc: Strum, Madeleine; Palma, Ted

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Ex. 6 - Personal Privacy

Please forward this information on to your States.

Thanks.

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morris.mark@epa.gov

To: Casso, Ruben[Casso.Ruben@epa.gov]
Cc: Palma, Ted[Palma.Ted@epa.gov]
From: Strum, Madeleine
Sent: Wed 7/15/2015 5:00:44 PM
Subject: chloroprene risk question

Ruben,

Given this plant has been emitting high levels of this pollutant for decades and is nearby a school (at least it looks that way) are there any data indicating any health issues in that area?

Madeleine Strum
U.S. Environmental Protection Agency
Office of Air Quality Planning and Standards/Air Quality Assessment Division/EIAG
919 541 2383 (voice)
919 541 0684 (fax)

To: Doris.B.Grego@dupont.com[Doris.B.Grego@dupont.com]
Cc: Casso, Ruben[Casso.Ruben@epa.gov]; Palma, Ted[Palma.Ted@epa.gov]; Thurman, James[Thurman.James@epa.gov]; Kelly.Petersen@LA.gov[Kelly.Petersen@LA.gov]
From: Strum, Madeleine
Sent: Wed 7/15/2015 4:48:12 PM
Subject: RE: Chloroprene Concentration

Hi Doris

I have found out the below information on the unit risk estimate. Although the emissions were about the same in 2011 as they were in the 2005 NATA (confirming what you said), this issue never came up because there wasn't a URE for chloroprene at that time.

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Madeleine Strum
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919 541 0684 (fax)

From: Doris.B.Grego@dupont.com [mailto:Doris.B.Grego@dupont.com]
Sent: Wednesday, July 15, 2015 12:29 PM
To: Strum, Madeleine
Subject: Chloroprene Concentration

Madeleine, what is the chloroprene concentration being used to determine the risk?

Thanks,

Doris B. Grego, P.E.

Senior Environmental Consultant

985-536-5437



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To: Thurman, James[Thurman.James@epa.gov]; Casso, Ruben[Casso.Ruben@epa.gov]; Palma, Ted[Palma.Ted@epa.gov]
From: Strum, Madeleine
Sent: Wed 7/15/2015 3:15:09 PM
Subject: RE: conference call -- 9 central/10 eastern and adobe connect link
[chloroprene_2005nata.xlsx](#)

Ex. 6 - Personal Privacy

All-

Ex. 5 - Deliberative

For the 2005 NATA (<http://www.epa.gov/ttn/atw/nata2005/tables.html>)

Emissions were 125 tons (compared to 130 tons in 2011) and are based on the 2002 calendar year data collected as part of the Residual Risk and Technology (RTR) standards development. So, as Doris (plant engineer) said, emissions haven't changed over the years.

It looks like from the 2005 NATA chloroprene file that there was no URE for the 2005 NATA. So that explains why it didn't show up.

Ex. 5 - Deliberative

I've attached the emissions (extracted from the Louisiana MDB file) and risks (extracted from the chloroprene MDB file) based on 2005 NATA/

Ex. 5 - Deliberative

Madeleine Strum
U.S. Environmental Protection Agency
Office of Air Quality Planning and Standards/Air Quality Assessment Division/EIAG
919 541 2383 (voice)
919 541 0684 (fax)

From: Strum, Madeleine

Sent: Wednesday, July 15, 2015 7:31 AM

To: 'Doris.B.Grego@dupont.com'; Thurman, James; Kelly.Petersen@LA.gov; Casso, Ruben

Subject: conference call -- 9 central/10 eastern 919 541 4328 and adobe connect link

If we need to share our screens to look at any of the files together, I've reserved an adobe connect meeting.

Just click on the link:

Ex. 6 - Personal Privacy

this will open up an application where we can share our screens if we need to.

Talk to you soon,

Madeleine

From: Doris.B.Grego@dupont.com [<mailto:Doris.B.Grego@dupont.com>]

Sent: Tuesday, July 14, 2015 10:44 AM

To: Strum, Madeleine; Thurman, James; Kelly.Petersen@LA.gov

Subject: Chloroprene Emissions - DuPont

I have converted the attachments I sent previously (Release Point Diagram and Fans location) to pdfs and are attached. I'm also including a Fan Drawing showing three of the Poly Building walls. The fans on the west side are the intake fans, the ones on the east and south walls are the discharge ones. This drawing does not show the fans as they are today, but it might help visualize the building and its venting system.

-----from earlier-----

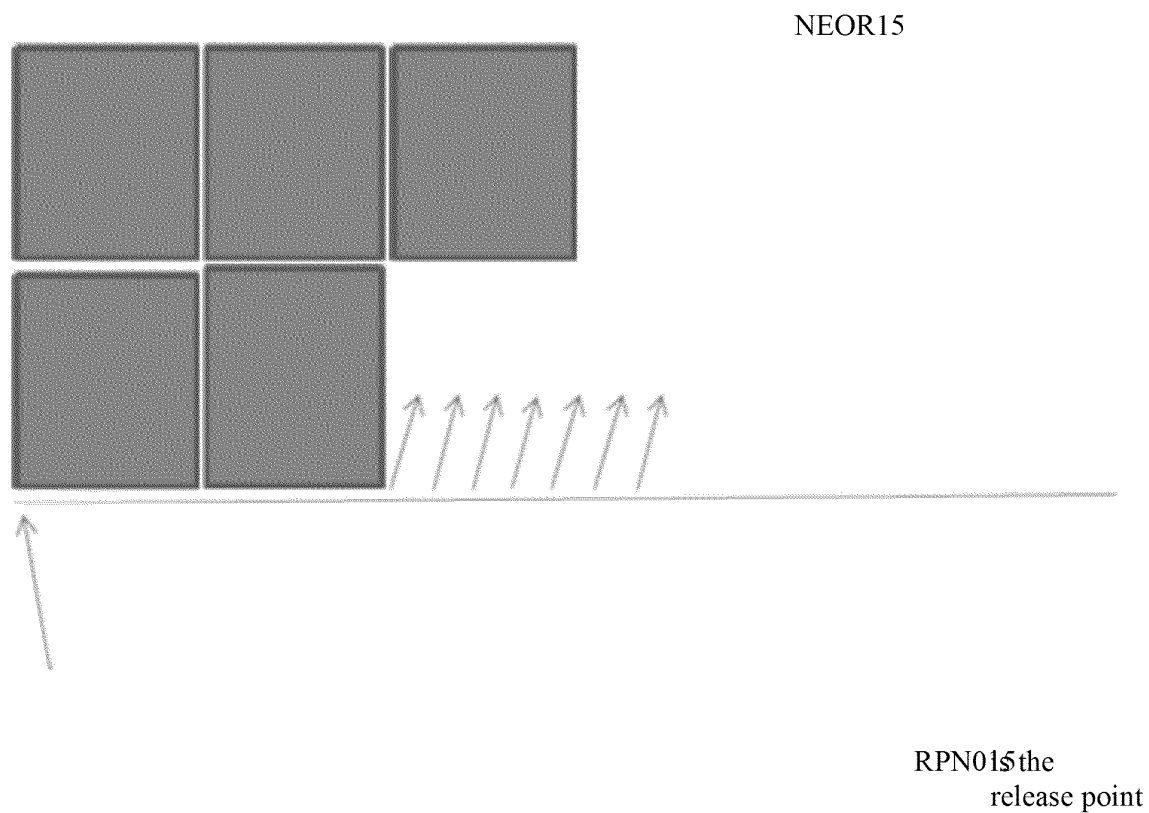
Attached is the revised EPA Modeling spreadsheet for the chloroprene sources at the DuPont Facility located in LaPlace, Louisiana. The changes are in red.

Two items need to be clarified.

1. On the chloroprene tab of the Modeling spreadsheet, the sources highlighted in pink do not discharge directly to the atmosphere, these sources are routed through one of the vents listed in rows 1 through 39.

For example sources NEO 222 thru 226 (rows 99 to 103) discharge through vent RPN015 which is source NEOR15 (row 1). Only the sources on rows 1 through 39 should be modeled.

See example below.



2. The second source on the spreadsheet, NEO185, consists of seventeen wall fans located on the Poly Building. Twelve fans are located on the east wall of the building, five are located on the south wall of the building. Attached is an Xcel file which includes two diagrams, one for each wall, and a table with the dimensions, emissions and locations of the fans. The fans are either 8' x 8' or 4' x 4', they are used to pull air from the building to minimize the concentration of chloroprene. For permitting and reporting purposes, I grouped all the fans into one fugitive emission source. For modeling purpose, they should be considered individually.

Doris B. Grego, P.E.

Senior Environmental Consultant

985-536-5437



From: Palma, Ted
Location: RTP-D201-Max40/RTP-Bldg-D
Importance: Normal
Subject: Accepted: Chloroprene Call with Denka/Dupont
Start Date/Time: Wed 12/9/2015 7:30:00 PM
End Date/Time: Wed 12/9/2015 8:30:00 PM

BEFORE THE ADMINISTRATOR
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

IN THE MATTER OF:)	
)	
)	
OPERATING PERMIT)	
CHLOROPRENE UNIT)	
DUPONT DOW ELASTOMERS, L.L.C.)	
LA PLACE, ST. JOHN THE BAPTIST)	PETITION NO. 6-03-02
PARISH, LOUISIANA)	
)	
Part 70 Operating Permit 3000-VO)	

ORDER DENYING PETITION FOR OBJECTION TO PERMIT

I. INTRODUCTION

On May 7, 2002, the Louisiana Department of Environmental Quality (“LDEQ”) issued DuPont Dow Elastomers, L.L.C., (“DuPont Dow”) a state operating permit for its Chloroprene Manufacturing Unit at its facility in La Place, St. John the Baptist Parish, Louisiana, pursuant to title V of the Clean Air Act, 42 U.S.C. §§ 7661-7661f, and its implementing regulations. *See* Permit 3000-VO (“title V Permit” or “permit”). The permit also constitutes a state preconstruction permit which authorized the replacement of a reactor system pursuant to the State’s minor new source review program. The Louisiana Environmental Action Network (“Petitioner” or “LEAN”) has requested that EPA object to the issuance of the title V permit pursuant to Section 505(b) of the Act and 40 C.F.R. § 70.8(d). Petition to Object (Nov. 13, 2001). Petitioner alleges that the permit is deficient on the ground that the emission limitations applicable to halogenated vent streams under 40 C.F.R. part 63, subpart G are not correctly determined in the permit.

II. STATUTORY AND REGULATORY FRAMEWORK

Section 502(d)(1) of the Act calls upon each state to develop and submit to EPA an operating permit program intended to meet the requirements of CAA title V. The State of Louisiana has a fully approved operating permit program which can be found at 40 C.F.R. part 70 Appendix A. Under these rules, major stationary sources of air pollution and other sources covered by title V are required to obtain an operating permit that includes emission limitations and such other conditions as are necessary to assure compliance with applicable requirements of the Act, including the applicable implementation plan. *See* CAA §§ 502(a) and 504(a).

The title V operating permit program does not generally impose new substantive air quality control requirements (referred to as "applicable requirements") on sources. The program does require permits to contain monitoring, recordkeeping, reporting, and other conditions necessary to assure compliance by sources with existing applicable requirements. *See* 57 Fed. Reg. 32250, 32251 (July 21, 1992). One purpose of the title V program is to “enable the source, States, EPA, and the public to better understand the requirements to which the source is subject, and whether the source is meeting those requirements.” *Id.* Thus, the title V operating permit program is a vehicle for ensuring that existing air quality control requirements are appropriately applied to a facility’s emission units in a single document, therefore enhancing compliance with the requirements of the Act.

Pursuant to Clean Air Act § 505(b)(2) and 40 C.F.R. § 70.8(d), if the EPA does not object to a facility’s draft title V operating permit on its own initiative, members of the public may petition the Administrator, within 60 days of the expiration of EPA’s 45-day review period, to object to the proposed permit. These sections also provide that a petition must be based only on

objections to the permit that were raised with reasonable specificity during the public comment period (unless the petitioner demonstrates that it was impracticable to raise such objections within that period or the grounds for such objections arose after that period).

Section 505(b)(2) of the Act requires the Administrator to issue a permit objection if a petitioner demonstrates that a permit is not in compliance with the requirements of the Act, including the requirements of 40 C.F.R. part 70 and the applicable implementation plan. In this case, the applicable requirements include 40 C.F.R. part 63, subpart G, one of the hazardous air pollutant (“HAP”) emission standards promulgated pursuant to Section 112(d) of the Act. If, in responding to a petition, EPA objects to a permit that has already been issued, EPA or the permitting authority will modify, terminate, or revoke and reissue the permit consistent with the procedures in 40 C.F.R. § 70.7(g)(4) or (5)(i) and (ii) for reopening a permit for cause. A petition for review does not stay the effectiveness of the permit or its requirements if the permit was issued after the expiration of EPA’s 45-day review period. *See* CAA § 505 (b)(2)-(b)(3); 40 C.F.R § 70.8(d).

III. BACKGROUND

On October 11, 1996, DuPont Dow submitted an application requesting a Part 70 operating permit for its Chloroprene Unit at the LaPlace, Louisiana facility. On November 14, 2000, DuPont Dow submitted a revision to the application to request authorization to replace the reactor system at the Chloroprene Unit with a new system that would have a higher conversion

rate and generate less waste.¹ The total amount of chloroprene produced per year would not increase due to the process modification. Title V Permit at 2.

The Chloroprene Unit has been in operation since before 1969. At the time of the application, it was covered by a state permit, Permit No. 3000, and several modifications thereto. The Chloroprene Unit is a Synthetic Organic Chemical Manufacturing Industry (“SOCMI”) facility and is a major source of regulated toxic air pollutants covered by, *inter alia*, 40 C.F.R. part 63, subpart G and L.A.C. 33:III.Chapter 51.

LDEQ published the proposed permit for public comment on August 25, 2001. LEAN submitted comments requesting, among other things, that additional information be made available to the public and that the comment period be extended. LDEQ published a second public notice announcing extension of the public comment period through December 5, 2001, and the scheduling of a public hearing on the same date.² LEAN submitted additional comments during the extended comment period. On May 7, 2002, LDEQ issued the final title V and preconstruction permit.

The emissions unit at issue in LEAN’s petition is the CD Vent Condenser (Emission Point No. 1110-4), which has a permitted emission rate for chloroprene of 18.3 tpy. *See* Petition at 1, 4; *see also* Title V Permit, Emission Inventory Questionnaire for No. 1110-4. LDEQ determined that the chloroprene emissions constituted a halogenated vent stream subject to 40 C.F.R. part 63, subpart G and classified it as a “Group 2” process vent based on the equation and requirements in

¹ DuPont Dow is awaiting issuance of a patent for the new reactor system before replacing the existing reactors. Title V Permit at 2.

² The hearing was adjourned when no public attendees were present.

40 C.F.R. § 63.115(d)(3) and the applicable coefficients listed in Table 1 to Subpart G. A Group 2 process vent is subject to the monitoring and reporting requirements set forth in 40 C.F.R. § 63.113(d) and (e), rather than the more stringent “Group 1” control requirements in § 63.113(a).

LEAN objects to the permit on the ground that LDEQ has misinterpreted 40 C.F.R. § 63.115(d)(3) and Table 1, and thus set the requirements for halogenated vent streams based on an incorrect Group 2 classification. Although LEAN styles its petition as raising five objections, all of the objections raise essentially this same issue. LEAN raised this issue in letters to EPA’s Region 6 office and the Office of Enforcement and Compliance Assurance (“OECA”) in 1996,³ and received responses from both offices explaining the Agency’s interpretation of 40 C.F.R. § 63.115(d)(3).⁴ LEAN’s five objections are: (1) LDEQ’s interpretation of § 63.115 is inconsistent with the Clean Air Act’s goal of protecting public health; (2) LDEQ’s interpretation would result in increased discharges of halogenated organic HAPs, posing risks to human health;⁵ (3) LDEQ’s interpretation results in greater controls of nonhalogenated vent streams relative to halogenated vent streams; (4) a rational interpretation of § 63.115 must result in a Group 1 classification and the accompanying control requirements; and (5) LDEQ has misinterpreted §

³ See Letter from M. Orr, LEAN, to S. Herman, EPA OECA (Aug. 29, 1996); Letter from M. Orr, to J. Saginaw, EPA Region 6 (Aug. 19, 1996).

⁴ See Letter from E. Stanley, EPA Office of Compliance, to M. Orr, LEAN (May 5, 1997) (“OECA Response Letter”); Letter from J. Luehrs, EPA Region 6, to M. Orr (Oct. 18, 1996) (“Region 6 Response Letter”).

⁵ Chloroprene is classified under State law as a Class II toxic air pollutant, and thus a “Suspected Human Carcinogen and Known or Suspected Human Reproductive Toxin.” L.A.C. 33.III.5112 Table 51.1.

63.115. EPA has performed an independent review of Petitioner's claims. Based on a review of all of the information before me, I hereby deny the Petition for the reasons set forth in this Order.

IV. EPA AGREES WITH LDEQ'S INTERPRETATION OF 40 C.F.R. § 63.115 AND FINDS IT CONSISTENT WITH SECTION 112 OF THE CLEAN AIR ACT.

Petitioner asserts that it is arbitrary and capricious for LDEQ to interpret 40 C.F.R. § 63.115 in a manner that allows halogenated organic HAPs such as chloroprene to avoid "Group 1" control requirements, and emphasizes the risk that this HAP poses to public health. However, the express terms of § 63.115(d)(3) govern and, in this case, result in a Group 2 classification. Petitioner's objection reflects a lack of understanding of the method by which air toxic standards are set under Section 112(d) of the Act.

Until 1990, the Clean Air Act required EPA to set risk-based air pollutant standards under Section 112 that would provide an "ample margin of safety to protect public health." *See Cement Kiln Recycling Coalition v. EPA*, 255 F.3d 855, 857 (D.C. Cir. 2001). To address problems with the implementation of risk-based regulation, Congress amended the Act in 1990 to require EPA to set technology-based standards, referred to as "maximum achievable control technology," or MACT standards. *Id.* at 858-59. EPA has implemented this requirement through a two-step process: the Agency first sets emission "floors" for HAP emissions from each source category, and then determines whether stricter standards are achievable in light of the factors listed in Section 112(d)(2), such as the cost-effectiveness of additional emissions reductions. *Id.* Congress recognized that risk to human health and the environment may remain under the technology-based approach, and reserved the development of standards where residual risk exists

for a second stage of regulation under Section 112(f), which is to occur “within 8 years” after Section 112(d) standards are promulgated.⁶

Subpart G, including 40 C.F.R. § 63.115, is a technology-based MACT standard promulgated under Section 112(d). Halogenated streams from process vents have certain treatment requirements according to whether they are determined to be a Group 1 or Group 2 stream under 40 C.F.R. § 63.111 and 40 C.F.R. § 63.115. The Group 1 or Group 2 classification depends, in part, on the Total Resource Effectiveness (“TRE”) index value, which is determined by the formula in § 63.115.⁷ The TRE index value serves as a measure of the supplemental total resource requirement per unit reduction of organic HAP emissions associated with the vent stream. *See* 40 C.F.R. § 63.111. In other words, “[t]he TRE is a decision tool that is used to determine if control of a process vent is required. The TRE is a standardized calculation that compares the annual cost of controlling a given vent stream with the emission reduction achieved.” *See* OECA Response Letter at 2. A process vent is classified as Group 1 if the TRE value is less than or equal to 1.0, and is classified as Group 2 if the TRE value is greater than 1.0

Section 63.115(d)(3) sets forth the formula for calculating the TRE. It further provides that the applicable coefficients from Table 1 of Subpart G shall be used in the formula as follows:

The owner or operator of a halogenated vent stream shall calculate the TRE index value based on the use of a *thermal incinerator with 0 percent heat recovery, and a scrubber*. The owner or operator shall use *the applicable coefficients in table 1 of this subpart for halogenated vent streams* located within existing sources. . . .

⁶ EPA is in the process of conducting the Section 112(f) review for the Synthetic Organic Chemical Manufacturing Industry standards.

⁷ The classification also depends on the vent stream flow rate and the total organic HAP concentration by volume. *See* 40 C.F.R. § 63.111. Those factors are not at issue in this petition.

Id. (emphasis added). Table 1 separates the appropriate coefficients for nonhalogenated vent streams from halogenated vent streams. The table, as it appears in the rule, is reprinted below, with the exception of the coefficients not at issue in this petition:

Type of Stream	Control Device Basis	Value of Coefficients			
		a	b	c	d
Nonhalogenated . .	Flare	1.935			
	Thermal Incinerator 0 Percent Heat Recovery	1.492			
	Thermal Incinerator 70 Percent Heat Recovery	2.519			
Halogenated	Thermal Incinerator and Scrubber	3.995			

The correct coefficient to use when determining the TRE for a halogenated stream is the coefficient listed under the heading “halogenated” for “Thermal Incinerator and Scrubber.” *See* OECA Response Letter at 2; Region 6 Response Letter at 1. This result is required by § 63.115(d)(3) which directs the source to use the “applicable coefficients in table 1 . . . for halogenated vent streams” and to use the coefficient based on the use of a “thermal incinerator with 0 percent heat recovery, and a scrubber.” There is only one entry for halogenated streams, and the control device basis listed for that stream, “Thermal Incinerator and Scrubber” plainly encompasses “a thermal incinerator with 0 percent heat recovery, and a scrubber.”⁸

This reading is consistent with the other provisions in 40 C.F.R. § 63.115(d). Section 63.115(d)(3)(ii) addresses the calculation of TRE index values for nonhalogenated streams. This

⁸ The Agency has previously explained that: “This equation for thermal incinerators with acid gas scrubbers was based on the 0 percent heat recovery scenario. This equation is based on the cost of controlling process vents using both a thermal incinerator and an acid gas scrubber used to remove acid gases created by combustion of the halogenated organic compound.” OECA Response Letter at 3.

provision shows that the three coefficients listed in Table 1 for nonhalogenated streams are to be used in determining the TRE for nonhalogenated streams, contrary to LEAN's suggestion that nonhalogenated stream coefficients also apply to halogenated streams. Specifically, § 63.115(d)(3)(ii) provides: "The owner or operator of a nonhalogenated vent stream shall calculate the TRE index value based on the use of a flare, a thermal incinerator with 0 percent heat recovery, and a thermal incinerator with 70 percent heat recovery and shall select the lowest TRE index value." Under the entry for nonhalogenated streams, Table 1 contains coefficients for each of these three control device bases - a flare, a thermal incinerator with 0 percent heat recovery, and a thermal incinerator with 70 percent heat recovery.

LEAN, however, argues that the correct coefficient to use for halogenated vent streams is the one listed under "Nonhalogenated" for "thermal incinerator with 0 percent heat recovery." Petition at 6. LEAN contends that the table is ambiguous and that "the two descriptions in the middle of the table ("Thermal Incinerator 0% Heat Recovery" and "Thermal Incinerator 70% Heat Recovery") are not limited by 'Type of Stream'" and thus must be used for halogenated streams. To address the inconsistency this interpretation would create with the requirement to base the TRE index value on use of a "thermal incinerator. . . and a scrubber" (§ 63.115(d)(3)), LEAN contends that a source must then calculate the TRE a second time -- a "second post-treatment calculation of the TRE" -- using the "thermal incinerator and scrubber" coefficient listed for halogenated streams. Petition at 6.

EPA disagrees with LEAN's interpretation. The table is not ambiguous. Under "Type of Stream" - "Nonhalogenated", there are three controls listed under "Control Device Basis." Under any rational reading of the table, the TRE coefficients apply only to the entry they follow --

nonhalogenated streams. Moreover, the regulation does not provide for a second “post-treatment” calculation of a TRE, and doing a post-treatment calculation makes no sense. The TRE index value is a measure of the cost-effectiveness of the potentially applicable control device – for halogenated streams, a thermal incinerator and scrubber – and is used to determine whether the stream must be controlled.⁹ OECA Response Letter at 3; Region 6 Response Letter at 1. The Agency’s response to comments in the Subpart G rulemaking reiterates this point. BID, Volume I, at 2-11 (“The TRE index value is a measure of cost-effectiveness of control and the TRE calculation for halogenated streams is based on application of a combustor followed by a scrubber.”) Thus, as the Agency has previously explained:

it is not correct to suggest that the halogenated category “thermal incinerator and scrubber” is for determining the TRE of a vent stream coming *out* of an incinerator The purpose of the acid gas scrubbers is to remove any acid gases created in the combustion of the process vent stream and is not intended to achieve greater control of emissions from the process vents.

OECA Response Letter at 4.

LEAN complains that the coefficient for halogenated streams is unreasonably high, making a Group 2 classification more likely and thus allowing halogenated organic HAPs to avoid the more stringent Group 1 control requirements. Petition at 7. However, it would be expected that the coefficient for halogenated streams is higher because halogenated streams would be subject to both a thermal incinerator and scrubber, in contrast to nonhalogenated streams, which would be

⁹ The Agency’s response to comments for the Subpart G rule reflect the function of the TRE index value in more detail: “The economic feasibility of controlling a vent stream is determined by the TRE calculation. The EPA has attempted to identify streams with high or ‘unreasonable’ cost-effectiveness through the establishment of a Group 1/Group 2 classification based either on TRE or on low flow and low concentration levels.” Docket No. A-90-19, Background Information Document (“BID”), Volume I at 2-22 (Mar. 9, 1994) (available at: <http://www.epa.gov/ttn/caaa/t3/reports/honbid1.pdf>).

subject to only a single control device (flare or incinerator). Two control devices predictably affect the cost-effectiveness rating, resulting in a higher TRE.

To summarize, 40 C.F.R. § 63.115(d)(3)(iii) clearly requires the TRE index value for halogenated vent streams to be determined based on the use of a thermal incinerator with 0 percent heat recovery and a scrubber. The correct coefficient is that listed in Table 1 under “Type of Stream - Halogenated,” across from “thermal incinerator and scrubber” – that is, 3.995. This results in a TRE value greater than 1.0 for the CD Vent Condenser.¹⁰ Therefore, it is a “Group 2 process vent” under § 63.111. As LEAN acknowledges, Group 2 process vents are subject to monitoring and reporting requirements under § 63.113(d), but not the control requirements of § 63.115(a). Additionally, the title V permit imposes conditions on the operation of the CD Vent Condenser as a recovery device to limit the chloroprene emissions and maintain a TRE index value above 1.0. Title V Permit, Specific Condition No. 2 & EIQ Sheet No. 1110-4.

¹⁰ LEAN concedes that use of the 3.995 coefficient yields a TRE index value greater than 1.0. *See* Petition at 7; Public Comments Response Summary for the Title V Permit, at 6 (TRE is 2.110). EPA has reviewed the TRE calculations and reached substantially the same TRE number. Using the equation in § 63.115(d), the TRE index value for the CD vent condenser is calculated as follows:

$$\begin{aligned} \text{TRE} &= (1/E_{\text{hap}}) [a + b(Q_s) + c(H_t) + d(E_{\text{toc}})] \\ \text{TRE} &= 0.5249 [3.9950 + 0.0039 - 0.0089 + 0.0018] \\ \text{TRE} &= 2.095 \end{aligned}$$

Coefficients from Table 1 to 40 C.F.R. part 63, subpart G: a = 3.995; b = 0.052; c = -0.001769; d = 0.00097

Q_s	H_t	E_{toc}	E_{hap}
0.0748 Dry scm/min	5.0276 Mega Joules/scm	1.9056 Kilograms/hr	1.9053 Kilograms/hr

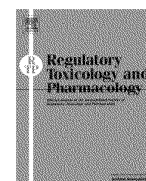
LEAN's real disagreement is with the merits of the standards set for process vents under 40 C.F.R. part 63, subpart G, and the exclusion of risk-based factors from Section 112(d). These are not valid grounds for objecting to a title V permit.

V. CONCLUSIONS

For the reasons set forth above and pursuant to Section 505(b) of the Act and 40 C.F.R. § 70.8(d), I deny the petition submitted by the Louisiana Environmental Action Network.

_____/s/
Michael O. Leavitt
Administrator

Date: 11/20/03



A constrained maximum likelihood approach to evaluate the impact of dose metric on cancer risk assessment: Application to b-chloroprene



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abstract

b-Chloroprene (2-chloro-1,3-butadiene, CD) is used in the manufacture of polychloroprene rubber. Chronic inhalation studies have demonstrated that CD is carcinogenic in B6C3F1 mice and Fischer 344 rats. However, epidemiological studies do not provide compelling evidence for an increased risk of mortality from total cancers of the lung. Differences between the responses observed in animals and humans may be related to differences in toxicokinetics, the metabolism and detoxification of potentially active metabolites, as well as species differences in sensitivity. The purpose of this study was to develop and apply a novel method that combines the results from available physiologically based kinetic (PBK) models for chloroprene with a statistical maximum likelihood approach to test commonality of low-dose risk across species. This method allows for the combined evaluation of human and animal cancer study results to evaluate the difference between predicted risks using both external and internal dose metrics. The method applied to mouse and human CD data supports the hypothesis that a PBK-based metric reconciles the differences in mouse and human low-dose risk estimates and further suggests that, after PBK metric exposure adjustment, humans are equally or less sensitive than mice to low levels of CD exposure.

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1. Introduction

b-Chloroprene (CD, CAS# 126-99-8, 2-chloro-1,3-butadiene) is a compound used in the manufacture of polychloroprene rubber. Chronic inhalation studies in animals have demonstrated that CD is carcinogenic in B6C3F1 mice and Fischer 344 rats in multiple target organs (lung, liver, circulatory systems, forestomach, Harderian gland, kidney, mammary gland, mesentery, oral cavity, skin, and thyroid gland) (Melnick et al., 1999; National Toxicology Program, 1998). In addition, respiratory and liver cancers have been associated with CD exposure in several epidemiological

studies (Acquavella and Leonard, 2001); however, interpretation of these findings has been difficult due to methodological limitations, including the inability to assign quantitative values for CD exposures, the small number of observed outcomes, and the small sample sizes for occupational studies (Marsh et al., 2007a). This makes the comparison of estimates of risk based on animal versus human results difficult.

While epidemiological studies are available for chloroprene, due to the uncertainties in the epidemiological studies the most recent quantitative risk assessment conducted by the USEPA (2010) used only animal data. The resulting cancer unit risk is driven by the most sensitive endpoint in animals, the incidence of lung tumors in female mice. Integration of the epidemiological studies does not provide compelling evidence for an increased risk of mortality from total cancers of the lung following inhalation exposure to chloroprene (Marsh et al., 2007a,b).

Previous studies have examined differences in toxicokinetics between animals and humans to determine if this is potentially

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the contributing factor to the differences in response between animals and humans. The initial step in metabolism is oxidation forming a stable epoxide, (1-chloroethenyl) oxirane, a genotoxicant that might be involved in the observed carcinogenicity in animals (Himmelstein et al., 2004b). Differences between the responses observed in animals and humans may be related to differences in toxicokinetics, to the metabolism and detoxification of potentially active metabolites (Himmelstein et al., 2004a,b), as well as to differences in species sensitivity. Specifically, Himmelstein et al. (2004a) found that the oxidation (V_{\max}/K_m) of CD in liver was slightly faster in rats and mice than in humans and hamsters, and in lung microsomes was much greater for mice compared to other species. In addition, hydrolysis (V_{\max}/K_m) of (1-chloroethenyl) oxirane, in liver and lung microsomes, was faster for humans and hamsters than for rats and mice.

In current risk assessments for chloroprene (USEPA, 2010), external exposure estimates are relied upon, which does not consider species differences in toxicokinetics. These differences may be critical in characterizing the potential risk of cancer following exposure to chloroprene, especially if the generation of a metabolite is related to the potential for cancer risk. The availability of physiologically based kinetic (PBK) models for both mice and humans (Yang et al., 2012) provides a unique opportunity for comparison of animal and human risk estimates based on external and internal exposure metrics. The PBK model for chloroprene incorporates the available data regarding species differences in metabolism of chloroprene. Application of the model allows for species-specific estimation of internal exposure metric, specifically the amount of chloroprene metabolized per gram of lung tissue. Risk estimates can then be compared across species based on this equivalent internal exposure metrics rather than external air concentrations.

The purpose of this study was to develop and apply a novel method that combines the results from available PBK models for chloroprene with a statistical maximum likelihood approach to test commonality of low-dose risk across species. This method allows for the combination of human and animal cancer study results to evaluate the difference between risk estimates obtained using both external and internal dose metrics.

The maximum likelihood approach applied allows for the evaluation of the ability of traditional dose–response models, such as the Multistage model, to describe the response pattern under the constraint of equal risk at a dose of interest (either internal or external), specifically a possible point of departure (POD). The results provide a demonstration of which dose metric provides statistically equivalent human- and animal-based risk estimates. Additional analyses were also conducted to investigate the impact of uncertainty in the estimated exposure levels for the human occupational study and to address the question of potential cross-species pharmacodynamic differences.

2. Material and methods

The method described here requires both animal data (a well-conducted two-year bioassay) and epidemiological data sufficient to allow dose–response analysis. Rather than modeling them separately, the approach adopted is to jointly model the selected studies to determine if, and under what circumstances, risk estimates of interest can be determined to be consistent across species. Jointly modeling the data requires software that allows for constrained maximization of the combined likelihood of the animal and human dose–response relationships with testing of hypotheses based on the comparison of the constrained maximum likelihood to the unconstrained (separate) likelihoods for the two species. Fig. 1 depicts the overall procedure.

2.1. Animal data

A two-year inhalation study of CD was conducted in F344/N rats and B6C3F₁ mice (National Toxicology Program, 1998). This is the bioassay relied upon by the Environmental Protection Agency (EPA) in the recent CD Integrated Risk Information System (IRIS) assessment (USEPA, 2010). Groups of 50 males and 50 females were exposed by inhalation for 6 h per day 5 days per week for 2 years to 0, 12.8, 32 or 80 ppm of CD. The National Toxicology Program (NTP) (1998) concluded that there was clear evidence of carcinogenicity in both the rats and mice following inhalation exposure to CD. In the F344/N rats, this conclusion was based on the increased incidences of neoplasms of the thyroid gland and kidney in males and females, increased incidences of neoplasms in the lung in males only and in the oral cavity and mammary gland in females only. In the B6C3F₁ mice, the conclusion of clear evidence of carcinogenicity was based on the increased incidence of neoplasms in the lung, circulatory system, forestomach and Harderian gland in both sexes, in the kidney for males only and the mammary gland, liver and skin for females only (see Table 5-4 in USEPA, 2010).

Based on the NTP (1998) results, USEPA (2010) concluded that that mouse is the most sensitive species, due to the increased tumor incidence and multisite distribution in the mouse relative to the rat. The EPA calculated a composite unit risk from all the female mice cancer endpoints listed above (9.8×10^{-1} per ppm; 2.7×10^{-4} per I g/m^3), and the unit risk estimated from the combined incidence of lung adenomas or carcinomas in the female mice produced the highest site-specific unit risk (6.4×10^{-1} per ppm; 1.8×10^{-4} per I g/m^3). As it was the most sensitive of the site-specific endpoints, combined lung adenomas and carcinomas is the endpoint considered in the current analysis. Analyses of rat responses, and perhaps additional mouse responses, may follow, given the success of this investigation.

2.2. Human data

Marsh et al. (2007a,b) conducted a historical cohort study to investigate the mortality of industrial workers potentially exposed to CD and other substances (including a potential confounding co-exposure to vinyl chloride). This study represents one of the most recent epidemiological studies and the design attempted to address the problems identified with earlier studies by conducting a detailed exposure assessment for both chloroprene and vinyl chloride monomer. The emphasis of the study was on cancer mortality, including respiratory system cancer. Four different CD production sites (i.e., Louisville, KY; Pontchartrain, LA; Maydown, Northern Ireland; and Grenoble, France) were included in the Marsh et al. study. The Louisville cohort examined by Marsh et al. (2007a,b) had the greatest number of exposed individuals, the greatest number of person-years of follow-up, and the greatest average exposure level (both in terms of the intensity level, ppm, and in terms of cumulative exposure, ppm-years). The greater exposure levels, combined with the greatest number of exposed individuals, increase the probability of detecting any carcinogenic effect following exposure to CD. Respiratory system cancer mortality from the Louisville cohort was used in this analysis as those data came from the best epidemiological dataset available (in terms of adequacy of size and suitability for dose–response analysis) that measured an endpoint that was comparable to the most sensitive endpoint in mice. The other cohorts may be subject to future analyses; inclusion of additional cohorts may increase the power of the epidemiological modeling.

For the Louisville cohort, approximate quartiles of the data were determined by Marsh et al. (2007b) based on the distribution of death from all cancers, and these quartiles were used to define

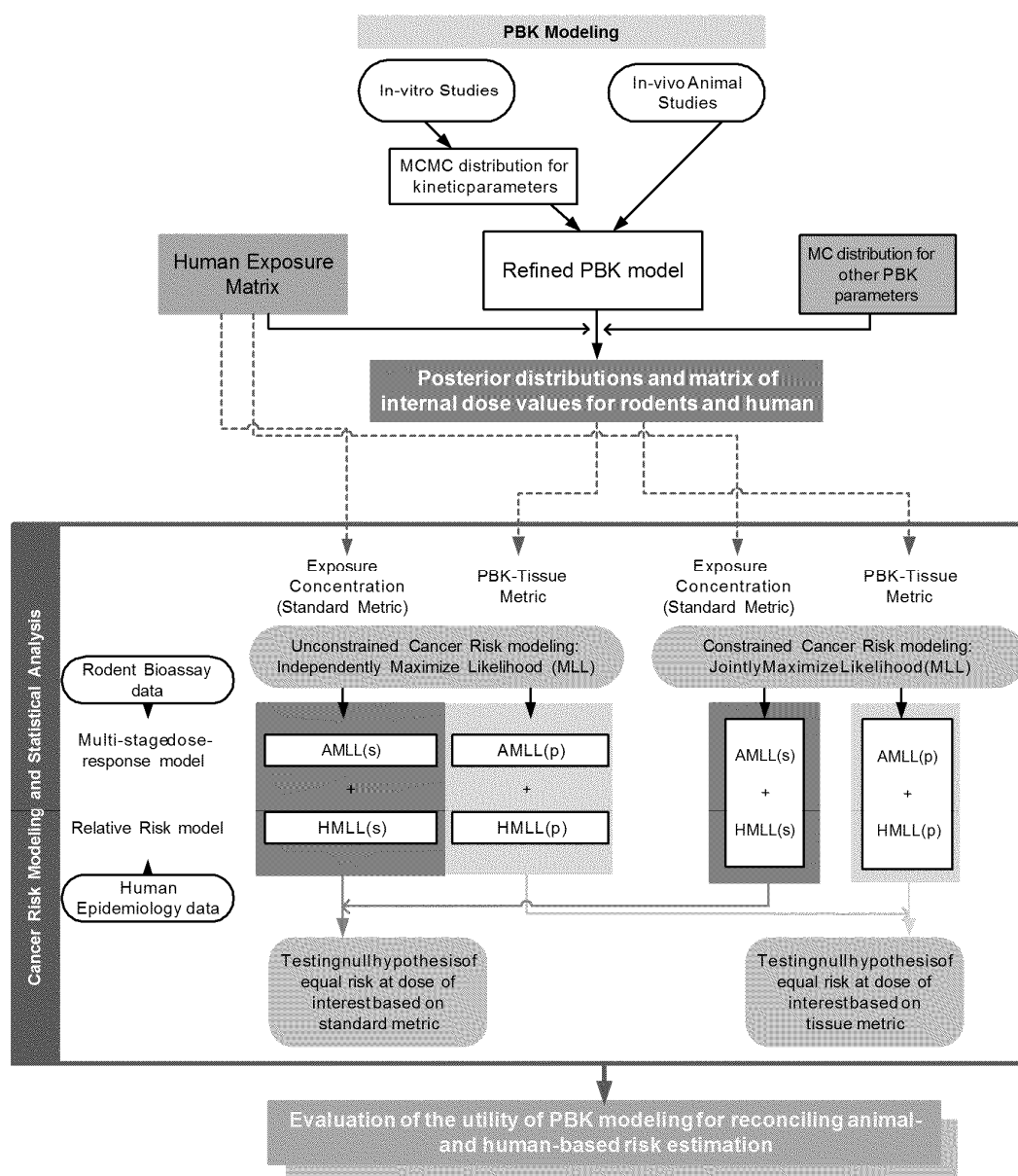


Fig. 1. Overview of physiological based kinetic modeling probabilistic dose response modeling.

the subgroups for all other cancer types, including the respiratory cancer used in this analysis. The exposure reconstruction detailed in Esmen et al. (2007b) was used, in combination with the Occupational Cohort Mortality Analysis Program (OCMAP) (described in detail in Marsh et al., 1998) to determine the quartile-specific and overall average cumulative exposure.

2.3. Estimation of exposure/dose

In the evaluation of the animal data, external air concentrations used in the exposure-response modeling were the administered air concentrations in the NTP (1998) study in ppm adjusted to an equivalent continuous exposure, adjusting for hours per day (6/24) and days per week (5/7) (Table 1). Similarly, the human cumulative doses were adjusted from occupational to continuous

exposure by adjusting for the number of work weeks per year (50/52), for work days per week (5/7) and for percentage of total daily inhalation that occurs during work hours (10/20) (USEPA, 2009). Adjusted values are shown in Table 2.

Based on the range of reported exposures for each quartile, the midpoints of cumulative exposure for the first three exposure groups were used (assumed to characterize the respective group average exposure for dose-response modeling). However, because the high exposure group was characterized as 164.053+ ppm-years with no highest exposure value, an approach was needed to characterize the average exposure for this group (Table 2). The average exposure used for the highest group was calculated based on the midpoint values for exposure groups 1 through 3, the overall average cumulative exposure computed by OCMAP, and the number of person-years apportioned to each group, shown here:

Table 1
Animal data modeled via the multistage model.

Dose group	Continuous exposure equivalent (ppm)	PBK metric (l mole/g-lung/day)	Group size	Number of animals with respiratory system cancer
1	0	0	50	4
2	2.3	0.705	49	28
3	5.7	1.12	50	34
4	14.3	1.47	50	42

Table 2
Human data modeled via a linear relative risk model.

Cumulative exposure group	Published cumulative exposure ranges (ppm-years)	Average cumulative exposure (ppm-years)	Assumed adjusted average cumulative exposure (ppm-years)	PBK metric (l mole of metabolite/g lung/day-years)	Person years of observation	Deaths from respiratory system cancer	SMR	Computed expected
1	<4.747	2.37	0.814	0.0083	68918	62	0.71	87.32
2	4.747–55.918	30.3	10.4	0.107	56737	67	0.71	94.37
3	55.918–164.052	110	37.8	0.387	39840	77	0.92	83.70
4	164.053+	297 ^a	102	1.05	32424	60	0.65	92.31

^a Calculated using text Eq. (1).

$$\text{ppm-years}_{\text{avg}}; \text{total} = \frac{1}{4} \sum_{i=1}^h \text{ppm-years}_{\text{avg}}; \text{total} = \frac{1}{4} \sum_{i=1}^h \text{PY}(i) = \text{PY}_{\text{total}}$$

where ppm-years(avg, total) is the average cumulative exposure for the entire cohort (80.35 ppm-years), ppm-years(avg, i) is the assumed average cumulative exposure for groups 1–3 or the unknown X ppm-years for group 4; PY(total) is the total number of person years of follow-up for the cohort (197919); and PY(i) is the person years of follow-up for group i (68918, 56737, 39840, and 32424 years for groups 1 through 4, respectively). The values for the ppm-year ranges and person years of follow-up (see also Table 2) are from Marsh et al. (2007b). The only unknown in the equation above, X, is for the ppm-years for group 4. Solving for X gives an estimate of the cumulative exposure for group 4 of 297 ppm-years.¹

An internal dose metric (PBK metric) was estimated for both the animal and human datasets using the PBK model by Yang et al. (2012). Following Markov Chain Monte Carlo (MCMC) analyses, Yang et al. derived a set of posterior distributions for each of the kinetic parameters in both the mouse and the human PBK models. The mean from each distribution (i.e., one for each kinetic parameter) as well as the standard physiological and partition coefficient values (Yang et al., 2012) for each species were used in the corresponding PBK model to derive the internal dose metric of l moles of metabolized CD/g lung/day for each exposure group in both the mouse experimental study and the human occupational study. Such a metric reflects the estimated metabolism of CD to reactive metabolites, including (1-chloroethenyl) oxirane, which are the proposed carcinogenic moieties (Yang et al., 2012). Since metabolism of CD is different between mice and humans, the use of PBK model estimates of internal dose, as a measure of exposure, provides a method to account for these species-specific differences.

For both the mouse and the human, the models were run for a week-long exposure (5 days per week). It was observed that after the 2 (weekend) days of non-exposure, chloroprene was cleared

¹ This approach used to determine the average concentration for the highest exposure group was deemed preferable to using a midpoint between 164 ppm-years and 1351.5 ppm-years, the reported maximum seen in the cohort. The dose for the highest group would have been larger (758 ppm-years) and would not have maintained the reported average ppm-year value for the entire cohort. Rather than relying upon a midpoint of the range of exposure, the consideration of average values for grouped exposure summaries in the current approach reflects all of the available information regarding cohort exposure.

from the body for both species. Thus, a single week of modeling the experimental exposures or occupational exposures was sufficient to calculate the lifetime daily average.

2.4. Calculation of animal-based risks

For the current assessment, the Multistage model provided in the USEPA Benchmark Dose Software (BMDS) program (USEPA, 2012) was fit to the female mice lung adenoma or carcinoma incidence data using the continuous exposure equivalent in ppm (adjusted from 6 h per day 5 days per week to continuous). In addition, the model was also fit to the data using the internal PBK metric of l mole CD metabolized/g of lung/day obtained from simulations of the Yang et al. (2012) PBK model (Table 1).

The multistage model has the mathematical form:

$$P(d) = 1 - e^{-[q_0 + q_1 d + \dots + q_k d^k]}$$

where d is the average lifetime daily dose, P(d) is the lifetime probability of tumor from the dose level d, and q₀, . . . , q_k are nonnegative parameters estimated by fitting the model to experimental animal data. The multistage modeling performed in this analysis assumed k = 2, i.e., it used a two-stage model.

The multistage model is a flexible statistical model that can describe both linear and non-linear dose-response patterns. It has been used as the standard for cancer risk analysis, and for many years the default dose-response model for federal and state regulatory agencies in the United States for calculating quantitative estimates of low-dose carcinogenic risks from animal data (USEPA, 1986, 2005).

The choice of a low-dose extrapolation method used by the EPA, in particular, in dose-response assessments should be informed by the available information on the mode of action of cancer, as well as other relevant biological information, and not solely on goodness-of-fit to the observed tumor data (USEPA, 1992). However, when data are limited or when uncertainty exists regarding the mode of action, models which incorporate low-dose linearity are the default approach. EPA usually employs the linearized multistage procedure in the absence of adequate information to the contrary; many of the available IRIS values are based on the results from this model. In that capacity, it is regularly used on data sets with only a few data points as is common for animal studies.

Using the external and internal dose metrics for CD, a single maximized log-likelihood was determined for each: the

unconstrained animal maximum log-likelihood for the standard (or external) metric (AMLLs) and the unconstrained maximized log-likelihood for the internal metric (AMLLp) (Fig. 1). Each of the AMLLx values represents the usual data-specific measure of the fit of the model to the animal bioassay results and is the maximum value of that log-likelihood with no other constraints.

2.5. Calculation of epidemiology-based risks

A linear relative risk model was fit to the summarized data from the Louisville cohort used in this analysis (Table 2).² The assumed average cumulative exposure, the observed deaths from respiratory system cancer, and the expected deaths from respiratory cancer were used in a linear model to estimate the relative risk:

$$\text{Relative Risk} \approx \text{Observed} - \text{Expected} \approx a \cdot d + b \quad (3)$$

where d is a measure of cumulative exposure and a and b are parameters to be estimated. “Expected” was computed as the observed number of cases (“Observed”) divided by the Standardized Mortality Ratio (SMR). Fitting to the human epidemiological data (Table 2) was accomplished via Poisson maximum likelihood techniques (Frome, 1983). The log-likelihood for the assumed Poisson distribution in a group having cumulative exposure d is expressed as:

$$LL \approx -\text{Expected} \approx a \cdot d + b - \ln(\text{Expected} \approx a \cdot d + b) \quad (4)$$

This log-likelihood ignores terms that are constant for the data set (i.e., do not depend on the values of the parameters). The maximum total log-likelihood (summed over each exposure group) was obtained and retained for future computations, as HMLLs or HMLLP, corresponding to the unconstrained human log-likelihood for the standard and PBK metrics, respectively.

2.6. Human–animal comparison of chloroprene risk estimates

The current method was developed to test the null hypotheses that certain dose metrics would provide comparable risk estimates across species, specifically mice and humans. The approach was designed to determine if one or more of the selected dose metrics was consistent with the hypothesis that there was a common risk level (across species) associated with a dose or exposure pattern of interest. The alternative hypothesis, for a given dose metric, was that the risk at the dose of interest was not the same across species.

Preliminary analyses had suggested that the benchmark dose at the extra risk level of 0.10 (BMD10) from the multistage dose–response model was just slightly less than 1 ppm, so this air concentration was selected as a reasonable concentration for comparison of risk estimates across species. For the PBK metric comparison, a value of 0.00352 l mole of CD metabolized/g-lung/day was selected as the internal dose metric of interest as that was the value estimated with model simulations conducted at either 1 ppm via an occupational exposure scenario or with the adjusted continuous exposure equivalent of 0.33 ppm.

For the ppm metric (the standard metric), a single maximized log-likelihood was determined, the unconstrained animal maximum log-likelihood for the standard metric (AMLLs) (Fig. 3). For the PBK metric, the maximum log-likelihood (AMLLp) was computed in exactly the same manner, but using the PBK metric values

as the dose inputs (Table 1). Correspondingly, calculation of human relative risks was conducted by fitting the relative risk model (Eq. (3)) to the epidemiology data to define the dose–response relationship using both the standard metric (with maximum likelihood HMLLs) and the PBK metric (yielding HMLLP). Using the animal and human log-likelihood estimates, unconstrained joint log-likelihoods of observing both the animal bioassay results and the epidemiological results were computed. The joint log-likelihoods were defined as “Unconstrained” meaning that the human and animal results were computed independently of one another. The computed unconstrained joint log-likelihoods (UMLLs and UMLLP) were determined based on the animal and human maximized log-likelihoods:

$$\text{UMLLs} \approx \text{AMLLs} + \text{HMLLs} \quad (5)$$

$$\text{UMLLP} \approx \text{AMLLp} + \text{HMLLP} \quad (6)$$

i.e., the metric-specific summation of the corresponding animal and human maximized log-likelihoods.

Constrained log-likelihoods were also calculated based on the null hypothesis that the animal bioassay data and the epidemiology data would provide the same estimate of risk at the dose of interest (1 ppm or 0.00352 l mole of CD metabolized/g-lung/day, depending on the metric under consideration). A joint log-likelihood for the combined human and animal results was calculated, under the assumption of equal risks at the dose of interest. If this constrained joint log-likelihood was sufficiently close to (by a formal statistical test) the unconstrained joint log-likelihood, then the null hypothesis of equal risks at those dose values was accepted.

The constrained maximum likelihood of interest was computed by examining values of b in the relative risk model (Eq. (3)), within a range of b values extending from 0 to an upper limit sufficient (by visual inspection) to guarantee that the maximum joint constrained log-likelihood was attained. For a selected value of b , the value of a in Eq. (3) was derived that maximized the human log-likelihood. In addition, for any selected value of b , a lifetime extra risk was calculated using the life table method used by EPA and others (Federal Register, 2004; USEPA, 2002, 2011) (Appendix A). The reference population for the life table calculations was the entire US population with rates from 2008 for all causes and respiratory system cancers (CDC, 2011). Risk was computed up through age 85. The lifetime human extra risk (HER) for a selected constant exposure level (dose-of-interest, or DOI) was computed using the life table approach with the various estimates of b ; it was referred to as the HER(DOI).

Given the HER(DOI) value defined above, the multistage model was fit to the animal data with an added constraint, i.e., that the animal extra risk at the DOI, AER(DOI), equals the HER(DOI). The source code for the BMDS multistage model was modified (code supplied by the authors on request) to allow for such constrained optimization; it is not possible to do it with the BMDS models as they are distributed. The modification automates the following calculations. If AER(DOI) is set equal to HER(DOI), then the multistage fit to the animal data can be maximized under that constraint:

$$\text{HER(DOI)} \approx \text{AER(DOI)} \approx \frac{1}{4} \left(\frac{1}{1 + e^{q_1 \cdot \text{DOI}}} + \frac{1}{1 + e^{q_2 \cdot \text{DOI}^2}} \right) \quad (7)$$

where the second equality follows from the form of the multistage model equation (Eq. (2)). Solving for q_1 , results in the following equation.

$$q_1 \approx \frac{1}{4} \ln \left(\frac{\text{AER(DOI)} - \frac{1}{4} \left(\frac{1}{1 + e^{q_2 \cdot \text{DOI}^2}} \right)}{\text{DOI}} \right) \quad (8)$$

Consequently, when AER(DOI) is fixed at a value, HER(DOI), the optimization for estimating the maximum (constrained) likelihood

² Even though the individual data for this cohort were available to the authors, we have used the summary data in order to demonstrate how this approach can be implemented with data that are commonly available when using epidemiological study reports for risk assessment. If we had used the individual data, we could, for example, have used a Cox proportional hazards model to better control for other variables, like age.

from the multistage model can be accomplished by varying q_0 and q_2 . (i.e., all the parameters other than q_1) and then computing q_1 as shown. For the current investigation, a 2nd degree multistage model was the highest polynomial degree needed. The same assumptions would apply for a polynomial degree greater than 2.

The two log-likelihood components, human and mouse, were then summed:

$$CMLLx \delta b \frac{1}{4} HMLLx \delta b \frac{1}{4} AMLLx \delta b \frac{1}{4} \delta 9 \frac{1}{4}$$

indicating the dependence on the choice of b . The value of “ x ” in Eq. (9) was either s (for the standard, ppm metric) or p (for the PBK metric), just as for the unconstrained likelihood calculations. The full range of allowable b values was examined to determine a maximum for $CMLLx(b)$; that maximum was the maximum constrained log-likelihood, $CMLLx$.

A likelihood ratio test was used to test the null hypothesis that the constraint of equal risks at DOI was true. The test statistics were:

$$2^{-1} \delta UMLLx \frac{1}{4} CMLLx \frac{1}{4} \delta 10 \frac{1}{4}$$

(twice the differences in the log-likelihoods, $x = s$ or p). There is one degree of freedom associated with the chi-squared distribution that approximates the distribution of those test statistics (Eq. (8) demonstrates there is one less parameter to be estimated, i.e., q_1 , when the constraint of $HER(DOI) = AER(DOI)$ is in effect, that is, when the null hypothesis is true). Larger differences in the maximized likelihoods yield larger values of the test statistic and therefore smaller p -values (i.e., probabilities of being in the tail of the chi-squared distribution to the right of the test statistic value). Small p -values (less than 0.05) were indicative of the null hypothesis being false.

2.7. Uncertainty analyses

An uncertainty analysis was conducted to evaluate the potential impact of the assignment of CD exposure concentrations (ppm) to the workers in the Louisville cohort. Esmen et al. (2007a) assigned nominal exposure levels to the members of the Louisville cohort, depending upon job class and calendar year. The uncertainty in the nominal levels was considered using “subtitles” for jobs within job class, the type of rotation among workers within those subtitles, and the deciles of the varying exposure levels associated with those subtitles. A Monte Carlo analysis was conducted, generating 3000 simulated human data sets, to evaluate the impact of exposure uncertainty. Each simulated human data set assigned different ppm exposure levels to each worker’s work history, consistent with exposure uncertainty distributions defined in the Supplemental material; a detailed description of the approach used in the Monte Carlo for the assigning of exposures concentrations to the workers is provided in that Supplemental material.

Given the rules specified in the Supplemental material, 1500 alternative (simulated) exposure histories for the cohort members were generated and run through the OCMAP program (Marsh et al., 1998). The output of each of those runs was a set of dose–response data analogous to those shown in Table 2. The cut points for defining the exposure groups were the same as used in the original analysis (Marsh et al., 2007b) (second column of Table 2).

When considering the PBK metric for humans, the above procedure was used to generate another set of 1500 simulated data sets, but an additional step was included to represent the uncertainty between the ppm exposure level and the PBK dose metric value. That additional step utilized the posterior distributions of the PBK model parameters derived by Yang et al. (2012). Following the assignment of each ppm exposure level as described in the Supplemental material, a PBK metric value was generated by sampling from a lognormal distribution with (natural scale) mean and coefficient of variation equal to,

Table 3
Heuristic for comparing models via Bayesian Information Criteria (BIC) values.

DBIC ^a	Strength of evidence
< 10	Very strong evidence for model i
10 to 16	Strong evidence for model i
16 to 22	Positive evidence for model i
22 to 28	Not much evidence either way
28 to 34	Positive evidence against model i
34 to 40	Strong evidence against model i
> 40	Very strong evidence against model i

^a DBIC = BIC(i) – BIC(j), where BIC(k) is the BIC associated with model k. Based on the categorization shown in Kass and Raftery (1995).

$$I \frac{1}{4} 0.00373^3 \text{ ppm} \delta 11 \frac{1}{4}$$
$$CV \frac{1}{4} 0.74; \delta 12 \frac{1}{4}$$

respectively. Those values for I and coefficient of variation (CV) (the log-scale variance equals $\ln[1 + CV^2]$) were selected based on the following observations. The posterior distributions of the PBK model parameters (Yang et al., 2012) were sampled 500 times each for five exposure concentrations ranging from 0.016 to 160 ppm (by factors of 10)³ and the associated PBK metric values (for the occupational exposure scenario) were computed for each sampling. As discussed elsewhere, the human ppm-to-PBK metric conversion is linear (for this range of ppm exposure levels); the factor of 0.00373 was associated with the average of the 2500 generated PBK metric values. Similarly, a CV of 0.74 was consistent with the variation observed across all those generated PBK metric values (conditional on the value of the mean).

The cut points on cumulative PBK metric values used to assign person years of observation to four exposure groups were those shown in Table 2 (second column) multiplied by 0.00352 (the conversion factor obtained when using PBK model parameter values equal to the means of each posterior distribution).

For each of the 3000 simulated data sets, the unconstrained and constrained maximization of the log-likelihoods was completed just as described in Section 2.5 above. For interpretation of the results of the uncertainty analysis the Bayesian Information Criteria (BICs) were used to evaluate the strength of the evidence for or against any given model. The BIC is defined as,

$$BIC \frac{1}{4} 2^{-1} MLL \frac{1}{4} \ln \delta n \frac{1}{4} \frac{1}{4} \text{parms}; \delta 12 \frac{1}{4}$$

where MLL , is the maximized log-likelihood, n is the number of observations, and parms is the number of parameters in the model. For the joint log-likelihoods (across mouse and human data sets) that we are analyzing here, $n = 8$ (four dose groups each for the mice and humans); $\text{parms} = 5$ for the unconstrained model (mouse and human data fit separately and independently) and $\text{parms} = 4$ for the constrained model (see Eq. (7) and associated text for a discussion of the reduction in the number of parameters under the constraint of equal risk at the DOI).

Lower values of the BIC indicate a better model. The BIC (like other information criteria) “rewards” a model for better fit (greater log-likelihood) but “penalizes” a model that uses more parameters to achieve a better fit. Put another way, the BIC rewards fit and parsimony.

A model comparison heuristic was introduced by Jeffreys (1961) and refined by Kass and Raftery (1995) (Table 3); it provides a categorization of the strength of the evidence for or against a given model, relative to another model. In our case, DBIC was defined with the unconstrained model as the referent, $DBIC = BIC$

³ These exposure levels were those reported in Esmen et al. (2007a,b) as the nominal chloroprene levels for their exposure classes (see their Table 2).

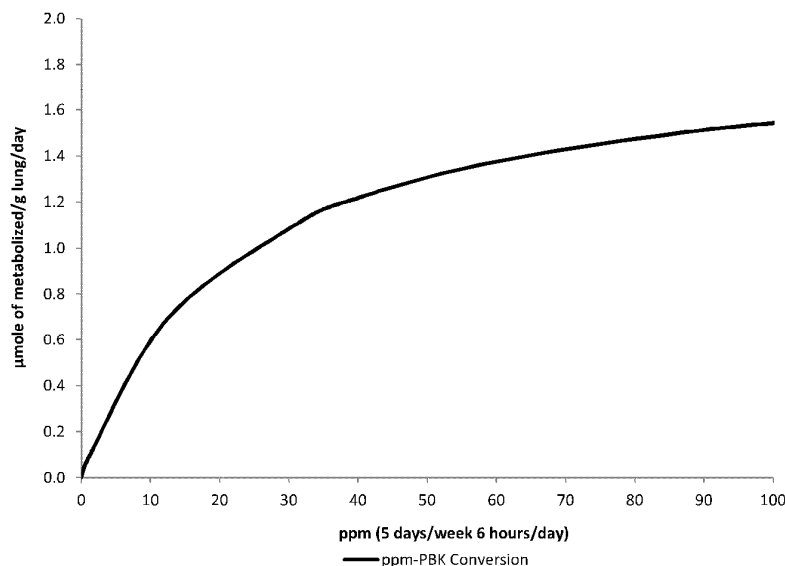


Fig. 2. Relationship between experimental exposure levels and PBK metric values; female mice.

(constrained) χ^2 BIC (unconstrained). Therefore, negative values of the DBIC favor the constrained model; positive values favor the unconstrained model. The results of the uncertainty analysis were summarized by tabulating the number of iterations of the simulations for which the constrained model falls in each of the evidence categories.

3. Results

The animal data set (Table 1) was not well described by the multistage model, when the doses were expressed in terms of the ppm exposure levels. The p-value for goodness-of-fit was 0.0046, a p-value indicating inadequate fit of the model to the data (p-values of greater than 0.10 are considered an adequate fit (USEPA, 2005)). The use of the PBK dose metric resulted in an adequate fit of the multistage model to the animal data (p-value = 0.44). Because of the saturation of metabolism in the lungs of female mice within the range of the experimental exposures (Fig. 2), the use of the internal PBK dose metric better correlated with the lung tumor incidence in the mouse than the external ppm dose metric. The PBK transformation was successful with respect to making differences in delivered dose accord with differences in response rates, when a multistage model represents the underlying carcinogenic process for the selected respiratory system cancer response.

The unconstrained, maximized log-likelihoods for the animal models were AMLLs = 105.758 (for the standard, ppm metric) and AMLLp = 101.049 (when using the PBK metric). The increase in the log-likelihood with use of the PBK metric is also indicative of a better fit, relative to use of the ppm exposure levels.

The human dose–response data (Table 2), were best fit by a relative risk model (Eq. (3)) with a slope (b) of zero and $a = 0.74$. The fact that $b = 0$ is consistent with the absence of a dose–response relationship between cumulative exposure and respiratory system cancer deaths in those workers.⁴ This was true whether or not the dose was expressed in terms of ppm-years or (l mole/g lung/day)–years,

at least partially because the PBK transformation in humans was linear for the relatively low exposure levels experienced by this cohort (Fig. 3). The maximized log-likelihood for the relative risk model with 0 slope was HMLLs = HMLLp = 849.396 (regardless of the dose metric used).

Therefore, the “base case,” unconstrained maximized combined log-likelihoods were,

UMLLs χ^2 743:638

613D

UMLLp χ^2 748:347

for the ppm exposure metric and for the PBK metric, respectively (Table 4).

3.1. Human–animal comparison of chloroprene risk estimates

The constrained optimization considered the animal and human data simultaneously, and maximized the sum of the animal and human log-likelihoods subject to one constraint, that the extra risk for the two fitted models be the same at the DOI. For the ppm exposure metric, the maximum constrained log-likelihood was attained when the relative risk slope was $b = 0.0017$ (per ppm-year). For that slope estimate, HMLLs(b) = 848.345, AMLLs(b) = 118.063 and therefore CMLLs = 730.282 (Table 4). The comparison of the constrained maximum log-likelihood to the unconstrained maximum log-likelihood (UMLLs = 743.638) indicates a statistically significant difference (p-value = 2.7×10^{-7}). This indicates that the animal- and human-based risks at 1 ppm are not the same (i.e., rejection of the null hypothesis). For the PBK metric, the DOI was set to 0.00352 l mole of CD metabolized/g lung/day, the PBK dose-metric that corresponds to an occupational exposure of 1 ppm. Under the constraint that the animal extra risk was the same as the human extra risk at that dose, the maximum constrained log-likelihood was attained when the relative risk slope was $b = 0.125$ (per (l mole/g lung/day) – years), and HMLLp(b) = 848.676, AMLLp(b) = 101.254 , and therefore CMLLp = 747.422.

The PBK metric provides consistent cross-species low-dose risk estimates (the p-value for the test of the null hypothesis equals 0.17). The null hypothesis of equal risk at the PBK dose of 0.00352 l mole/g lung/day would not be rejected at the typical

⁴ For the relative risk model, the slope was constrained to be non-negative. No evaluation was conducted to determine if negative values for the slope were better than zero. It was considered implausible that chloroprene exposure would reduce respiratory cancer risk.

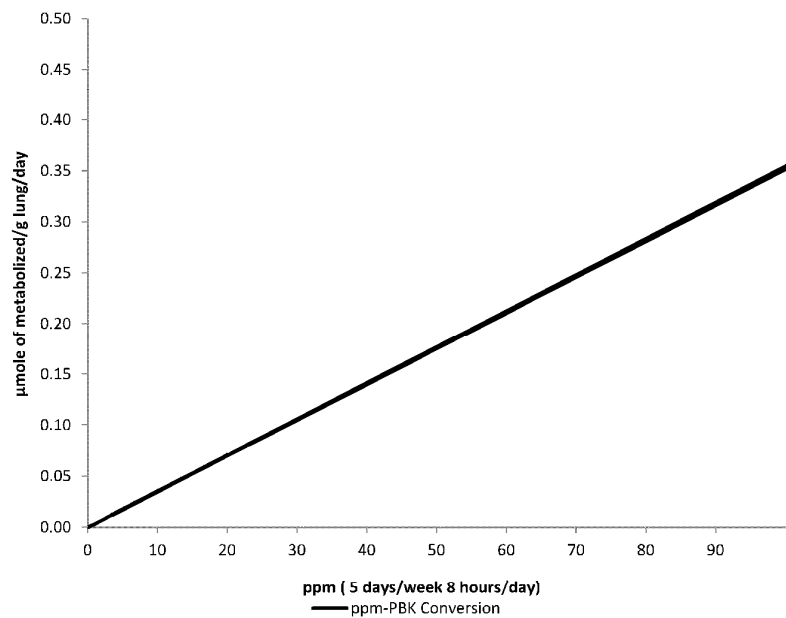


Fig. 3. Relationship between occupational exposure levels and PBK metric values; humans.

0.05 level of significance. Not only did the PBK transformation of doses result in a substantially improved model fit to the animal

Table 4
Unconstrained and constrained maximized log-likelihoods.

Dose-metric	Animal	Human	Combined
Unconstrained			
ppm metric	105.758	849.396	743.638
PBK metric	101.049	849.396	748.347
Constrained			
ppm metric	118.063	848.345	730.282
PBK metric	101.254	848.676	747.422

data, it also reconciled cross-species predictions of risk estimates for low doses.

Naturally, the unconstrained fit to the animal data provided the best fit. Although the constrained fit to the animal data (where the animal risk at the DOI was constrained to equal the human risk at the DOI) was not as good as the unconstrained fit, the predicted probabilities of response were still well within the (1 SE) error bars associated with the observed response rates (Fig. 4). Importantly, the constrained curve had a less steep slope at low doses, which conforms better to the (at most) shallow slope for the human dose–response. The achievement of a shallow low-dose slope with enough curvature to match the observations at the higher

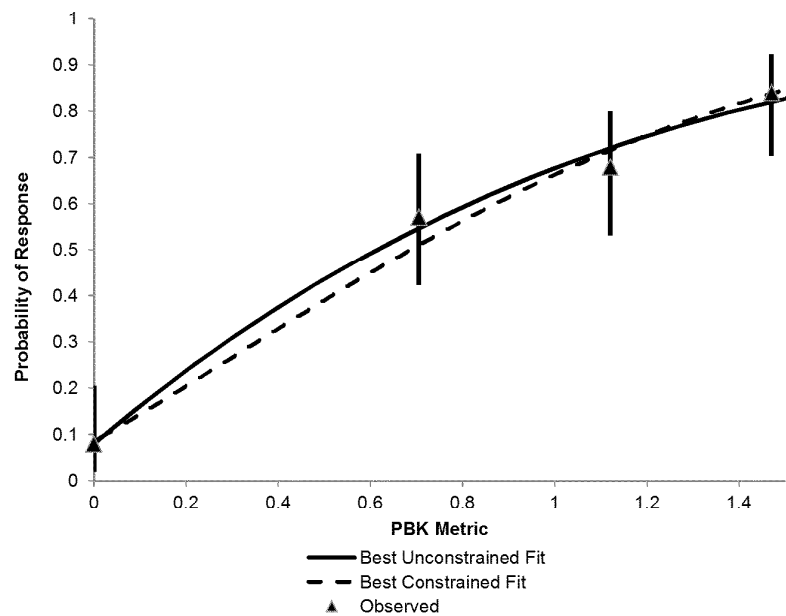


Fig. 4. Comparison of best unconstrained and constrained fits to animal data.

Table 5
Evidence for and against the constrained model, by exposure metric.^b

DBIC ^a	Strength of evidence	No. simulated cohort data sets in each category	
		ppm metric	PBK metric
< 10	Very strong evidence for constrained model	0	736
10 to 16	Strong evidence for constrained model	1	284
16 to 22	Positive evidence for constrained model	16	236
22 to 28	Not much evidence either way	46	162
28 to 6	Positive evidence against constrained model	131	63
6 to 10	Strong evidence against constrained model	259	13
>10	Very strong evidence against constrained model	1047	6

^a DBIC = BIC(constrained) - BIC(unconstrained).

^b Each simulated cohort data set was subject to constrained and unconstrained maximum likelihood estimation. The final two columns shows the number (out of 1500) of those data sets that had different degrees of support for or against the constrained model, depending on the choice of exposure metric.

experimental exposure levels is what allows for a consistent risk estimate at the DOI.

3.2. Uncertainty analyses

Uncertainty in estimated human exposures had an interesting effect on the comparison of the constrained and unconstrained models (Table 5). For the models applied to the ppm metric, exposure uncertainty implied a range of estimates that predominantly did not support the constrained model; all but 63 (of 1500) simulated exposure runs demonstrated evidence against the constrained model and, therefore, against the hypothesis that mice and humans have equal risk at 1 ppm (when risks were equilibrated on the basis of ppm exposure levels). When the PBK metric was used, there was a notable shift to values that favor the constrained model. A total of 1256 runs demonstrated evidence for the constrained model (nearly half were consistent with very strong evidence in favor of the constrained model and, therefore, for the equality of animal and human risks at low doses). The ability to eliminate one parameter in the optimization was of key importance, especially when the log-likelihoods for the constrained and the unconstrained models were similar. The DBIC for the base case (no uncertainty) constrained model using the PBK metric was -0.23 , i.e., little or no evidence for or against it relative to the unconstrained model. This result is consistent with the failure to reject the null hypothesis of no difference in risk across species at the PBK dose of interest.

4. Discussion and conclusions

The analysis described here presents a new method to compare and test risk predictions across species for lifetime extra cancer risk. It requires that specific methods be applied as appropriate to the type of data available, but all having the goal of predicting lifetime extra cancer risk. Thus, for the epidemiological data,

relative risk Poisson modeling linked to life-table calculations yields the necessary risk estimates. For the animal bioassay data, multistage modeling is applied. Those two sides of the analysis were subject to a formal statistical evaluation that addressed hypotheses of interest using likelihood procedures.

This approach allows for reproducible and consistent comparisons of experimental and/or observational data that are commonly used for risk assessment purposes. In the specific case of CD, the results of applying this approach indicate that external, concentration-based estimates of exposure to CD are not the appropriate dose metric for estimating comparable risk estimates across species. Even when accounting for one of the largest uncertainties associated with the use of epidemiological data for dose–response assessment, i.e., reconstructing occupational human exposure levels, there was little or no statistical support for the hypothesis that human and animal low-dose risks are equivalent when exposure was expressed in terms of ppm air concentration. Conversely, the use of the PBK metric, daily amount of CD metabolized at the target per gram of tissue, in the dose–response models provided better fit of the models to the data due to the ability of the PBK metric to account for the cross-species metabolic differences. It also resulted in comparable risk estimates across species at the dose of interest, and more generally, at all doses less than or equal to the dose of interest.

The evaluation of the animal and human data using the PBK metric provided cancer slope factors between 2.9×10^{-5} and 1.4×10^{-2} per ppm, with the maximum-likelihood estimate of 6.7×10^{-3} per ppm. The human equivalent cancer slope factor estimated based on the incidence of lung tumors in female mice (the most sensitive sex and species) reported in the EPA Toxicological Review (2010) is 6.5×10^{-1} per ppm (adjusted for exposure 6/24 h and 5/7 days). This slope factor is approximately 100 times greater than the maximum-likelihood estimate determined with the current approach.

While the current adjustment for pharmacokinetic differences across species results in comparable risk estimates, there are

Table 6
Evaluation of the presence of pharmacodynamic differences across species.

Relative pharmacodynamic sensitivity	Mouse PBK metric value (1 mole of CD metabolized/g lung/day)	Mouse metric/human metric	Test of equality of risks at the specified PBK doses (p-value) ^a
Humans more sensitive	0.0845	24	0.001
	0.0282	8	0.029
	0.00845	2.4	0.056
Humans equally sensitive	0.00352	1	0.17
Humans less sensitive	0.00282	0.8	0.22
	0.000845	0.24	0.54

^a P-values are from the test of various null hypotheses, i.e., that the risk at the specified mouse metric values is equal to the risk at the human PBK metric value of 0.00352 1 mole/g lung/day (the constrained maximum likelihood calculations). The alternative hypotheses are that there is no such constraint; the mouse and human models are independent so do not necessarily predict equivalent risks at the specified doses.

additional factors that could be considered to further refine the evaluation. These could include species-specific differences in detoxification and pharmacodynamics.

In the case of CD, the data are not currently available to estimate or model the magnitude of species differences in such additional factors. However, the current analysis approach provides evidence that, if and when such data become available they will demonstrate that humans are equally or less sensitive, but not more sensitive than mice, at the low levels of CD exposure investigated. That “working hypothesis” results from the analysis results shown in Table 6. If one assumes that risk is equal when the human PBK metric value is 0.00352 l mole CD metabolized/g-lung/day and the mouse metric value is at different levels (greater or less than 0.00352), equivalence of risk was only supported (having p-values greater than 0.05) when the proposed equivalent-risk mouse dose was less than or equal to about 2.4 times the human dose of 0.00352. The working hypothesis of lower human low-dose risk still remains to be tested formally with data specifically obtained and appropriate for that purpose. Until then, the results of the current analyses suggest that humans are equally or less sensitive than mice to equivalent low-dose CD exposures.

Conflict of interest

B.C. Allen: Sub-contract to ENVIRON International Corporation and The Hamner Institutes for Health Sciences. C. Van Landingham: Contract to ENVIRON International Corporation. Y. Yang: Personal fees from International Institute of Synthetic Rubber Produces, Inc. (IISRP). A.O. Youk: Grants from International Institute of Synthetic Rubber Produces, Inc. (IISRP) and personal fees from DuPont Chemical Company. G.M. Marsh: Grants from International Institute of Synthetic Rubber Produces, Inc. (IISRP), personal fees from DuPont Chemical Company. N. Esmen: Nothing to disclose. P.R. Gentry: Contract to ENVIRON International Corporation. H.J. Clewell III: Personal fees from International Institute of Synthetic Rubber Produces, Inc. (IISRP). M.W. Himmelstein: Nothing to disclose.

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Appendix A. Formulae for calculating extra risk using a life-table method

The probability of disease occurrence (incidence or mortality) between ages x_1 and x_2 may be expressed as:

$$p_{D0D} = \int_{x_1}^{x_2} h(x) S(x) dx \quad \text{A1}$$

where $S(x)$ is the probability of survival to age x given survival to age x_1 and $h(x)$ is the instantaneous hazard of disease occurrence at age x . This integral can be approximated by a sum:

$$p_{D0D} \approx \sum_{i=1}^n p(i) S(i) \quad \text{A2}$$

where the age interval $[x_1, x_2]$ has been divided into n subintervals with the i th subinterval having width $D(i)$, $i = 1, \dots, n$, $p(i)$, representing the probability of disease occurrence in the i th age interval, is calculated as:

$$p(i) = q_c(i) D(i) \quad \text{A3}$$

and $S(i)$, representing the probability of surviving to the beginning of the i th age interval given survival to age x_1 , is calculated as $S(1) = 1$ and:

$$S(i) = \exp\left(-\sum_{j=1}^{i-1} q_a(j) D(j)\right) \exp\left(-\sum_{j=1}^{i-1} q_c(j) D(j)\right); \quad i > 1 \quad \text{A4}$$

where $q_c(i)$ and $q_a(i)$ are the cause-specific rate of occurrence and all-cause death rates for the i th age interval obtained from standard rate tables. An alternative to (Eq. (A4)) is given by:

$$S(i) = \exp\left(-\sum_{j=1}^{i-1} q_a(j) D(j)\right); \quad i > 1; \quad \text{A5}$$

which encompasses slightly different interpretations of the standard rates. These 2 expressions generally agree closely.

If the subintervals correspond to individual years, (Eqs. (A2) and (A4)) take on the simplified forms:

$$p_{D0D} = \sum_{i=x_1}^{x_2} q_c(i) S(i) \quad \text{A6}$$

and:

$$S(i) = \exp\left(-\sum_{j=x_1}^{i-1} q_a(j)\right) \exp\left(-\sum_{j=x_1}^{i-1} q_c(j)\right) \quad \text{A7}$$

Once the background rates q_c and q_a are selected, these equations completely determine $p(0)$. These same formulae are used to calculate the probability of response, $p(D)$, from a particular exposure pattern, D , by replacing the rates q_c and q_a by the appropriate modification that accounts for the model-predicted effect of exposure on these rates. The appropriate modifications depend upon the form of the dose–response model estimated from the epidemiologic data, and the assumed exposure pattern. If the dose–response model predicts relative risk as a function of some exposure metric, then:

$$q_c(i) \text{ is replaced by } q_c(i) R(i) \quad \text{A8}$$

and:

$$q_a(i) \text{ is replaced by } q_a(i) R(i) \quad \text{A9}$$

where $R(i)$ is the relative risk predicted by the dose–response model, i.e., $R(i) = 1 + b \cdot D(i)$, where $D(i)$ is the cumulative dose at age i from exposure pattern D . The latter replacement involves subtracting from the total death rate the background death rate from the disease of interest, and adding back this contribution adjusted by the effect of exposure.

Once $p(0)$ and $p(D)$ have been calculated, the extra risk from exposure pattern D is computed as:

$$\frac{1}{p_{D0D}} \left(p_{D0D} - p_{D0D} \right) = p_{D0D} \quad \text{A10}$$

This extra risk is what will be compared with the animal-based extra risk estimate.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.yrtph.2014.07.001>.

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**Kinetic Modeling of β -Chloroprene Metabolism: Probabilistic in vitro-
in vivo Extrapolation of Metabolism in the Lung, Liver and Kidneys of
Mice, Rats and Humans**

SUPPLEMENTAL DATA

Yuching Yang, Matthew W. Himmelstein, Harvey J Clewell

Supplemental data

A. Supplemental text

A1. Detailed Microsomal Sample Preparation

A2. Description of Computational process for Markov-Chain Monte Carlo (MCMC) simulation

B. Exemplar model code

B1. AcslX code to describe the CD oxidation in the in-vitro system (PK model)

B2. AcslX code define the setting used for probabilistic analysis of CD in-vitro dataset

B3. Script to perform MCMC analysis of microsomal data (Rat liver as example)

C. Supplemental table and figures

C1. Summary of ANOVA results

C2. Comparison of tissues and gender-specific clearance for mouse and rat

C3. Probability frequency of chloroprene oxidative metabolism parameters

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Supplemental Data A: Supplemental text
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A.1 Detailed Microsomal Preparation

Female mice were 12.7 weeks of age at the time the liver and lung microsomes were prepared. Female rats were 10.9 weeks when liver and lung microsomes were made. For kidney microsomes, the male and female mice and rats were 11.9 weeks of age when the microsomes were made. Lung and liver microsomes were prepared by differential centrifugation as described by Himmelstein et al. (2004). The microsomal preparations were analyzed for protein by the Bradford (1976) method (Bio-Rad Laboratories, Hercules, California, U.S.A.). The P450 content was measured by spectrophotometry using established methods (Omura and Sato, 1964; Guengerich, 1982). All fractions were stored at -70°C . Stock protein were measured and used.

Human kidney microsomes were purchased from Xenotech (H0610.R, Lot No. 0810236, Lenexa, Kansas, U.S.A.). The preparation was a mixed pool (10 mg protein/mL) from 8 individuals representing 4 subjects per sex, 7 of which were Caucasian and one African American, with ages ranging from 48 to 69 years. The vendor characterized activity for NADPH-cytochrome c reductase and lauric acid 12-hydroxylation were 34.5 ± 0.3 and 0.820 ± 0.146 nmol/mg protein/min, respectively.

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A.2 Description of Computational process for Markov-Chain Monte Carlo (MCMC) simulation

The following steps, describing a generalized computational procedure during the MCMC iterations, are applicable to both hierarchical and population-only Bayesian analysis conducted in current study.

- | Step | Computation |
|------|---|
| A | Sample population parameter 'M' from the prior distribution |
| B | Sample gender-specific variability 'S' from the prior distribution |
| C | Sample gender-specific parameter 'm' from Norm (M, S) |
| D | Calculate metabolic parameter (Vmax, Km or Vmax/Km) as $\exp(m)$ |
| E | Compute the model predictions with the updated model parameters |
| F | Compute the posterior likelihood with each new updated parameter based on their prior distributions and the experimental data |
| G | Repeat steps D-F for each gender |
| H | Repeat steps A-G for each MCMC iteration until convergence of the posterior distributions of M and m is reached. |

--

Supplemental Data B: Exemplar model code

B1. AcsIX code to describe the CD oxidation in the in-vitro system (PK model)

Chloroprene PK model for microsomal data

!! : CSL file is the actual PK model file
program invitro.csl

VARIABLE TIME

INITIAL

```

CONSTANT VMAX1a=0.      !'MAX RATE OF MET. (uMOL/HR/mg protein)'
CONSTANT VMAX1b=0.      !'MAX RATE OF MET. (uMOL/HR/mg protein)'
CONSTANT KM1a=0.1       !'MICHAELIS CONSTANT (uMOL/L)'
CONSTANT KM1b=0.1       !'MICHAELIS CONSTANT (uMOL/L)'

CONSTANT VK=0.          !'REPRESENT THE V/K COEFFICIENT FOR RAT LUNG (1/hr)'
CONSTANT RLOSS=0.       !'REPRESENT THE background loss rate (1/hr)'
CONSTANT P1=0.69        !'MEDIA/AIR PARTITION for CD'

CONSTANT A10=0.         !'INITIAL AMOUNT IN VIAL (uMOL)'

CONSTANT VVIAL=0.01163  !'VOLUME OF VIAL (L); Vial volume= 11.65 ml'
CONSTANT VMED=0.001     !'VOLUME OF MEDIA (L); Liquid volume'
VAIR=VVIAL-VMED         !'HEADSPACE'
CONSTANT PROT = 1.0     !'AMOUNT OF PROTEIN (mg)'

CONSTANT TF=0.          !'TIME OF FIRST SAMPLE (hr); kept same'
CONSTANT TI=0.2         !'INTERVAL BETWEEN SAMPLES (hr)kept same'
CONSTANT VINJ=0.0002    !'INJECTION VOLUME (L); based on Matt email'

```

!'Initial Conditions'

```

CA10=A10/(VAIR+P1*VMED)
CM10=CA10*P1           !'CONC in SOLUTION'
CA1=CA10
CM1=CM10
A1I=0.

```

!'TIMING COMMANDS'

```

CONSTANT TSTOP=1.1      !'LENGTH OF EXPOSURE (HOURS)'
CONSTANT POINTS=100.    !'NO. OF POINTS IN PLOT'
INTERVAL CINT=0.01
TS=TF
SCHEDULE step .AT. TF

```

END !'END INITIAL'

DYNAMIC

ALGORITHM IALG=2

DERIVATIVE

TERMT(TIME.GE.TSTOP)

! 'CD KINETICS (umoles/hr)'

```

R1Ma=(VMAX1a*CM1)/(KM1a+CM1)*PROT
R1Mb=(VMAX1b*CM1)/(KM1b+CM1)*PROT
RRLUNGVK=VK*CM1
RRLOSS=RLOSS*CM1
A1Ma=INTEG(R1Ma,0.)
A1Mb=INTEG(R1Mb,0.)
ARLUNGVK=INTEG(RRLUNGVK,0.)

```

```
ARLOSS=INTEG(RRLOSS, 0.) !background loss rate
```

```
CA1=(A10-A1Ma-A1Mb-ARLUNGVK-A1I-ARLOSS)/(VAIR+VMED*P1)
```

```
CM1=CA1*P1
```

```
A1=CA1*VAIR+CM1*VMED
```

```
! 'MASS BALANCE'
```

```
CHECK1 = A10 - (A1+A1Ma+A1Mb+A1I+ ARLUNGVK+ARLOSS)
```

```
DISCRETE step
```

```
PROCEDURAL
```

```
!Routine for sample loss'
```

```
  A1I=A1I+CA1*VINJ
```

```
  SCHEDULE step .AT. TS+TI
```

```
  TS=TS+TI
```

```
END          !'END PROCEDURAL'
```

```
END          !'END DISCRETE'
```

```
END          !'END DERIVATIVE'
```

```
END          !'END DYNAMIC'
```

```
END          !'END PROGRAM'
```

B2. AcsIX code define the setting used for probabilistic analysis of CD in-vitro dataset

M-Script to perform MCMC analysis of microsomal data: MCMC setting and function

```

function tchains = runmcmc(pchains = [])
% Driver code for MCMC analysis
global zdata
global firstT
global lastT
global firstD
global lastD
global CCC
global LI
global sLV
global sLK
global Vmax
global Km
global sVmax
global sKm
global sVK
global preds
LI = zeros(1, 1);
sLV = zeros(1, 1);
sLK = zeros(1, 1);
Vmax = zeros(1, 1);
Km = zeros(1, 1);
sVmax = zeros(2, 1);
sKm = zeros(2, 1);

numParms = 9
numChains = 1
numIts = 2000000
funcNames = ["mcInIt", "mcEvalLikelihoods", "mcEvalPriors", "mcSamplePriors", "mcEvalProposal", "mcSampleProposal"]
updateMode = 4
chains = mcmc(numParms, numIts, numChains, updateMode, funcNames, pchains);
save @format=ascii @file=mcmc_results.dat chains
tchains = chains([1:50:2000000].:);
end

function mcInIt()
global zdata
global firstT
global lastT
global firstD
global lastD
global CCC
global LI
global sLV
global sLK
global Vmax
global Km
global sVmax
global sKm
global sVK
global preds
global OpMcmcPriorBounds
OpMcmcPriorBounds = [...
0.01, 10
0.01, 10
0.01, 10
-10, 5
-10, 5
-20, 10
-20, 10
-20, 10
-20, 10
-20, 10
];
global OpMcmcAdaptive
OpMcmcAdaptive = 1;
global OpMcmcDelayedRejection

```

```

OpMcmcDelayedRejection = 0;
global OpMcmcAdaptPeriod
OpMcmcAdaptPeriod = 30;
global OpMcmcAdaptCovarScale
OpMcmcAdaptCovarScale = 1;
global OpMcmcLoggingPeriod
OpMcmcLoggingPeriod = 50;
global OpMcmcAdaptLowerThresh
OpMcmcAdaptLowerThresh = 0.25;
global OpMcmcAdaptUpperThresh
OpMcmcAdaptUpperThresh = 0.45;
global OpMcmcAdaptLowerThreshDR
OpMcmcAdaptLowerThreshDR = 0.45;
global OpMcmcAdaptUpperThreshDR
OpMcmcAdaptUpperThreshDR = 0.65;
global OpMcmcSigmaDecreaseFact
OpMcmcSigmaDecreaseFact = 0.9;
global OpMcmcSigmaIncreaseFact
OpMcmcSigmaIncreaseFact = 1.1;
global OpMcmcDRSigmaReduceFact
OpMcmcDRSigmaReduceFact = 0.2;
global OpMcmcDRSigmaReduceFactAM
OpMcmcDRSigmaReduceFactAM = 0.1;
global OpMcmcAdaptLowerThreshAM
OpMcmcAdaptLowerThreshAM = 0.15;
global OpMcmcAdaptUpperThreshAM
OpMcmcAdaptUpperThreshAM = 0.3;
global OpMcmcCovarScaleDecreaseFact
OpMcmcCovarScaleDecreaseFact = 20;
global OpMcmcCovarScaleIncreaseFact
OpMcmcCovarScaleIncreaseFact = 20;
global OpDemcSnookerFraction
OpDemcSnookerFraction = 0.1;
global OpDemcThinningFactor
OpDemcThinningFactor = 10;
global OpDemcB
OpDemcB = 0.0001;
end

function samp = mcSampleProposal(prevsamp)
global zdata
global firstT
global lastT
global firstD
global lastD
global CCC
global LI
global sLV
global sLK
global Vmax
global Km
global sVmax
global sKm
global sVK
global preds
samp = [];
% This function is a stub...
% Code for a user-defined proposal function can be inserted here.
end

function val = mcEvalProposal(samp, prevsamp)
global zdata
global firstT
global lastT
global firstD
global lastD
global CCC
global LI
global sLV
global sLK
global Vmax

```

```
global Km
global sVmax
global sKm
global sVK
global preds
val = 0;
% This function is a stub...
% Code for a user-defined proposal function can be inserted here.
end
```

```
function mcDumpSamples()
global zdata
global firstT
global lastT
global firstD
global lastD
global CCC
global LI
global sLV
global sLK
global Vmax
global Km
global sVmax
global sKm
global sVK
global preds
LI
sLV
sLK
Vmax
Km
sVmax
sKm
end
```

```
function names = mcSampNames()
names = "LI";
names = [names, "sLV"];
names = [names, "sLK"];
names = [names, "Vmax"];
names = [names, "Km"];
names = [names, "sVmax(1)"];
names = [names, "sVmax(2)"];
names = [names, "sKm(1)"];
names = [names, "sKm(2)"];
names
end
```

```
function parms = mcPackSamples()
global zdata
global firstT
global lastT
global firstD
global lastD
global CCC
global LI
global sLV
global sLK
global Vmax
global Km
global sVmax
global sKm
global sVK
global preds
parms = [];
parms = [parms LI];
parms = [parms sLV];
parms = [parms sLK];
parms = [parms Vmax];
parms = [parms Km];
parms = [parms reshape(sVmax, 1, 2)];
```

```

    parms = [parms reshape(sKm, 1, 2)];
end

function mcUnpackSamples(parms)
    global zdata
    global firstT
    global lastT
    global firstD
    global lastD
    global CCC
    global LI
    global sLV
    global sLK
    global Vmax
    global Km
    global sVmax
    global sKm
    global sVK
    global preds
    idx = 1;
    LI = parms(idx); idx = idx + 1;
    sLV = parms(idx); idx = idx + 1;
    sLK = parms(idx); idx = idx + 1;
    Vmax = parms(idx); idx = idx + 1;
    Km = parms(idx); idx = idx + 1;
    sVmax = reshape(parms(idx:idx+1), 2, 1); idx = idx + 2;
    sKm = reshape(parms(idx:idx+1), 2, 1); idx = idx + 2;
end

function parms = mcSamplePriors()
    global zdata
    global firstT
    global lastT
    global firstD
    global lastD
    global CCC
    global LI
    global sLV
    global sLK
    global Vmax
    global Km
    global sVmax
    global sKm
    global sVK
    global preds
    LI = normrnd(1, 1);
    Vmax = unifrnd(-10, 5);
    Km = unifrnd(-10, 5);
    sLV = lognrnd(-1.2, 1.6);
    sLK = lognrnd(-1.2, 1.6);
    for gg = 1 : 2
        sVmax(gg) = normrnd(Vmax, sLV);
        sKm(gg) = normrnd(Km, sLK);
    end
    parms = mcPackSamples();
end

function val = mcEvalPriors(parms)
    global zdata
    global firstT
    global lastT
    global firstD
    global lastD
    global CCC
    global LI
    global sLV
    global sLK
    global Vmax
    global Km
    global sVmax
    global sKm

```

```

global sVK
global preds
mcUnpackSamples(parms);
val = 0.0;
val = val + normlpdf(LI, 1, 1);
val = val + uniflpdf(Vmax, -10, 5);
val = val + uniflpdf(Km, -10, 5);
val = val + lognlpdf(sLV, -1.2, 1.6);
val = val + lognlpdf(sLK, -1.2, 1.6);
for gg = 1 : 2
    val = val + normlpdf(sVmax(gg), Vmax, sLV);
    val = val + normlpdf(sKm(gg), Km, sLK);
end
end

function val = mcEvalLikelihoods(parms)
global zdata
global firstT
global lastT
global firstD
global lastD
global CCC
global LI
global sLV
global sLK
global Vmax
global Km
global sVmax
global sKm
global sVK
global preds
mcUnpackSamples(parms);
val = 0.0;
sVK = 0;
for gg = 1 : 2
    for i = firstD(gg) : lastD(gg)
        preds = getpreds(sVmax(gg), sKm(gg), sVK, CCC(i), gg);
        for j = firstT(gg) : lastT(gg)
            if(~isnan(zdata(j, i)))
                val = val + normlpdf(zdata(j, i), preds(j), LI);
            end
        end
    end
end
end
end
end

```

B3. Script to perform MCMC analysis of microsomal data (Rat liver as example)

```
load @format = model @file = /home/yyang/work/Chloroprene/ACSL/MCMC/RatBothLiver/chain1/invitro.so
```

```
prepare @clear
prepare @all
```

```
disp('Both Fisher Rat, Liver Case')
seedrnd(4556)
```

```
VVIALF=0.01165; %% Male ==VVIAL=.0119573;
VVIALM=0.0119573;
VMED=.001;
VINJF=0.0002; %% Male ==VIN=0.0003858 !important
VINM=0.0003858;
VAIRF=VVIALF-VMED;
VAIRM=VVIALM-VMED;
TSTOP=1.2;
TF=0.;
Ti=0.2;
PROT = 1.0;
P1 = 0.69;
WESITG=0;
WEDITG =0;
```

```
start @nocallback
```

```
global _ca1
global _time
global zdata
global tFindex
global tMindex
```

```
global firstT
global lastT
global firstD
global lastD
global CCC
global ControlData
```

```
use ('/home/yyang/work/Chloroprene/ACSL/MCMC/Control/ControlData.m')
```

```
%CDF Liver Summary
```

%Time	1 ppm	10 ppm	50 ppm	150 ppm	270 ppm
0.	0.052	0.465	1.935	6.243	11.007
0.2	0.015	0.141	0.844	4.460	9.091
0.4	0.006	0.048	0.360	3.274	7.661
0.6	0.003	0.022	0.188	2.479	6.621
0.8	0.002	0.011	0.103	1.958	5.831
1.	NaN	0.007	0.066	1.607	5.202

```
%[Time 264 ppm 132 ppm 50 ppm
```

%[Time	264 ppm	132 ppm	50 ppm
0	2.0125	4.6755	9.824;
0.025	2.18	4.503	9.454;
0.05	1.634	4.318	8.939;
0.1	1.354	3.918	9.767;
0.15	1.113	3.708	9.603;
0.2	0.893	3.217	7.856;
0.225	0.931	3.007	7.581;
0.25	0.706	2.885	7.02;
0.3	0.545	2.559	7.925;
0.35	0.419	2.478	7.679;
0.4	0.291	2.0245	6.097;
0.425	0.308	1.841	5.974;
0.45	0.237	1.786	5.568;
0.5	0.175	1.547	6.201;

0.55	0.125	1.558	NaN;
0.6	0.077	1.1375	4.637;
0.625	0.082	1.01	4.584;
0.65	0.067	0.995	4.231;
0.7	0.048	0.837	NaN;
0.75	0.034	0.708	NaN;
0.8	0.0195	0.5715	3.482;
0.825	0.02	0.483	3.428;
0.85	0.018	0.489	3.18;
0.9	NaN	0.397	NaN;
0.95	0.009	NaN	NaN;

```
tempF= size(FratFLiver);
tempM= size(FratMLiver);
```

```
ID_Time = 1;
ID_DoseF = [(ID_Time+1):1:tempF(2)];
ID_DoseM = [(ID_Time+1):1:tempM(2)];
```

```
dataF = FratFLiver(:, ID_DoseF);
dataM = FratMLiver(:, ID_DoseM);
```

```
tempF= size(dataF);
tempM= size(dataM);
```

```
tFindex = FratFLiver(:, ID_Time);
tMindex = FratMLiver(:, ID_Time);
```

```
% number of time points :max(tempM(1), tempF(1))
% number of dose : (tempM(2)+tempF(2))
```

```
zdata = NaN* ones([max(tempM(1), tempF(1)), (tempM(2)+tempF(2))]); % corresponde to max 25 timepoints and 5 dose each
gender
zdata(1:tempF(1), 1:tempF(2)) = dataF ;% first Female, then Male
zdata(1:tempM(1), tempF(2)+1:tempF(2)+tempM(2)) = dataM;
```

```
firstT = [1, 1];% time point;% first Female, then Male
lastT = [tempF(1), tempM(1)];
firstD = [1, tempF(2)+1];% dose groups% first Female, then Male
lastD = [tempF(2), tempF(2)+tempM(2)];
```

```
AAF=dataF(1,:)*(VAIRF+P1*VMED);
AAB=dataM(1,:)*(VAIRM+P1*VMED);
```

```
CCC = [AAF, AAB];
zdata=log(zdata);
```

```
function preds = getpreds(Vmax, Km,VK, A10, Gender)
    global _ca1
    global _time
    global tFindex
    global tMindex
    global ControlData
```

```
% draw back ground loss rate
tmp = ceil(rand*500);
lossR = ControlData(tmp);
setmdl("RLOSS", exp(lossR));
```

```
setmdl("VMAX1A", exp(Vmax)); % reset model parameter as global variables
setmdl("KM1A", exp(Km));
setmdl("VK", VK);
setmdl("A10", A10);
```

```
if Gender==1
    tindex = tFindex;
    setmdl("VVIAL", 0.01165);
    setmdl("VINJ", 0.0002);
```

```
else
    tindex = tMindex;
    setmdl("VVIAL", .0119573);
    setmdl("VINJ", 0.0003858);
end

data @clear
data("SAMPTIMES", ["T"], tindex);

start @nocallback

preds = NaN*ones(length(tindex), 1);

for i = 1:length(tindex)
    idx = find(_time == tindex(i));
    if(idx ~= [])
        preds(i) = max(0.0, _ca1(idx));
    end
end

preds = log(preds);

end

use invitromc11.m
chains = runmcmc();
```



Supplemental Data C: Tables and Figures

C1: Summary of ANOVA results

Table S1 presents the summary results of the Kruskal–Wallis one-way analysis of variance (ANOVA). Kruskal–Wallis ANOVA is a non-parametric method for testing whether samples originate from the same distribution. In this analysis, the null hypothesis is whether the posterior distributions of tissue-specific intrinsic clearance for male and female are the same for mice and rat. Table S2 is an example ANOVA outputs generated in Matlab.

Table S1: Summary of Kruskal–Wallis ANOVA results

	Rat Prob>Chi-Sq	Mice Prob>Chi-Sq
intrinsic clearance		
Liver	0	0
Lung	<0.0001	0
Kidney	0	0

Table S2: Kruskal-Wallis ANOVA TABLE of Intrinsic Clearance of Lung in Rat

Source	SS	dF	MS	Chi-sq	Prob>Chi-sq
Column	5.44E+09	1	5.43E+09	652.68	5.83E-144
Error	7.79E+10	9998	7.79E+06		
Total	8.33E+10	9999			

C2: Comparison of tissues and gender-specific clearance for mouse and rat

Figure S1. Comparison of Distributions of Gender-specific Intrinsic Clearance in Liver, Lung, Kidney for Mice

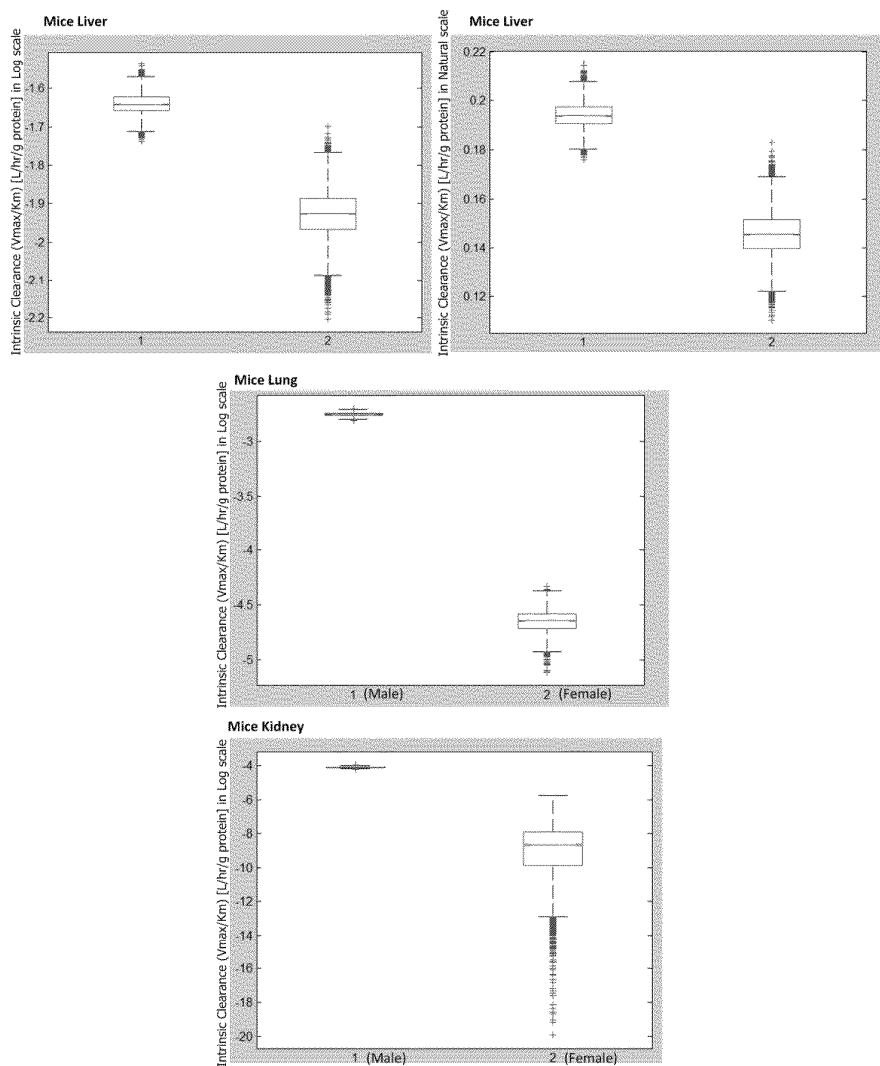
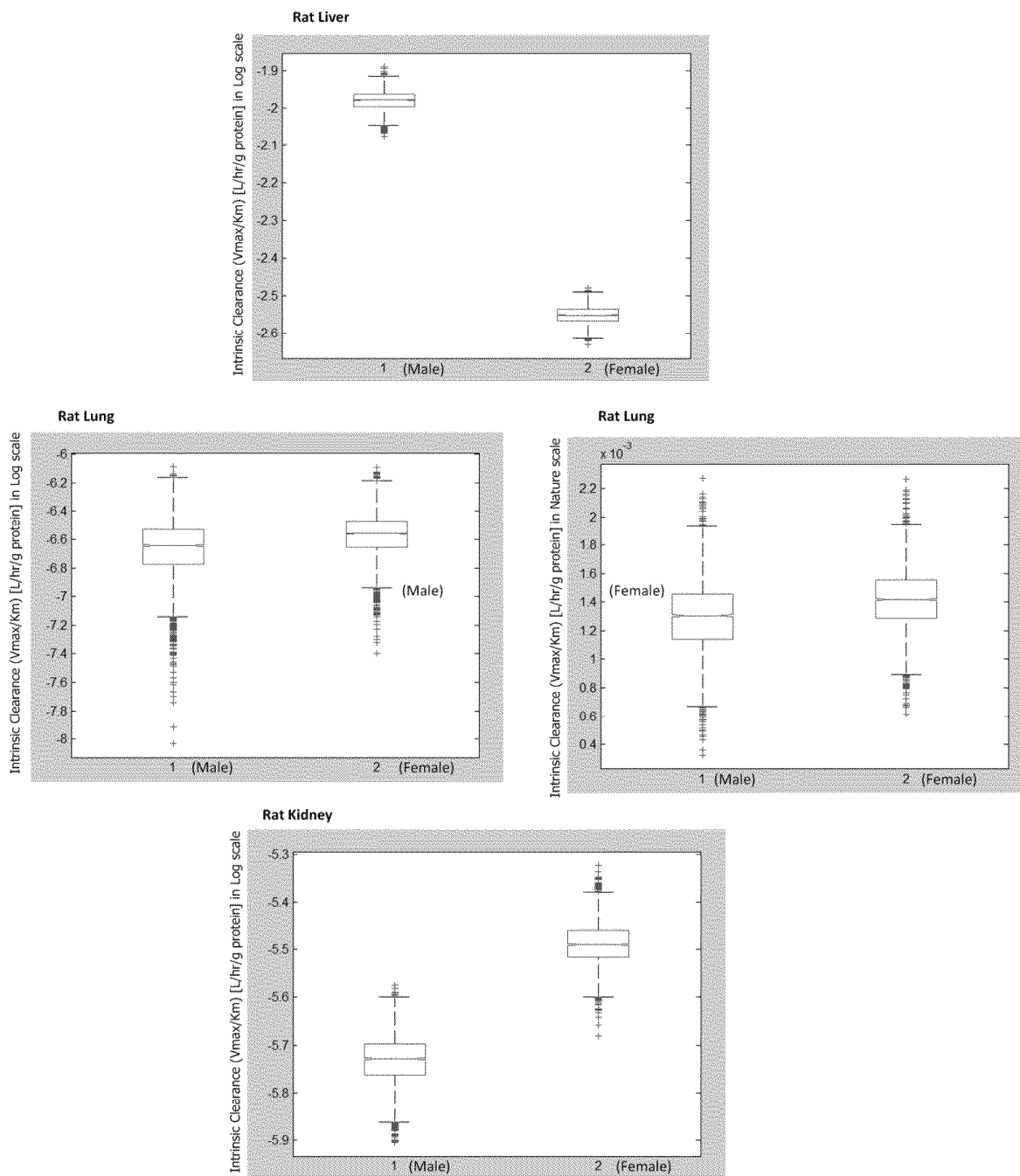
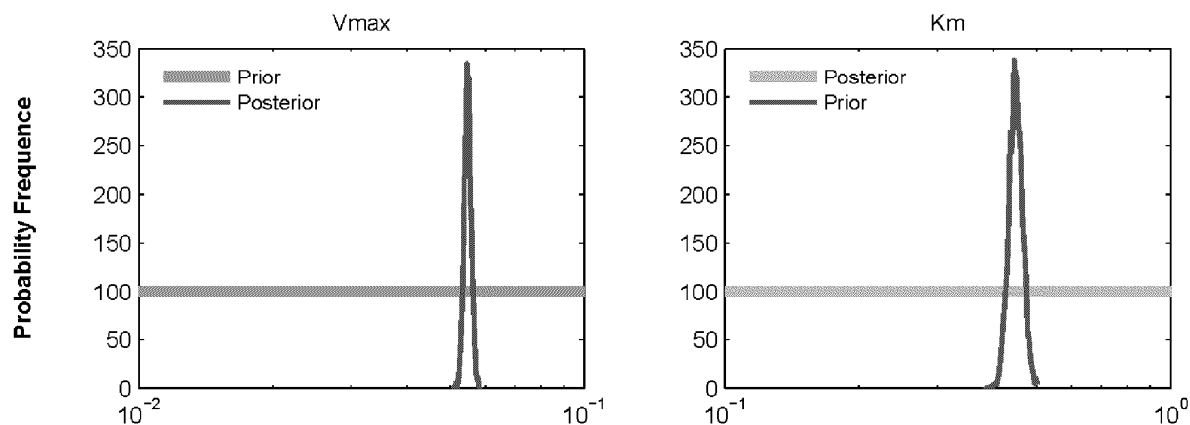


Figure S2. Comparison of Distributions of Gender-specific Intrinsic Clearance in Liver, Lung, Kidney for Rat



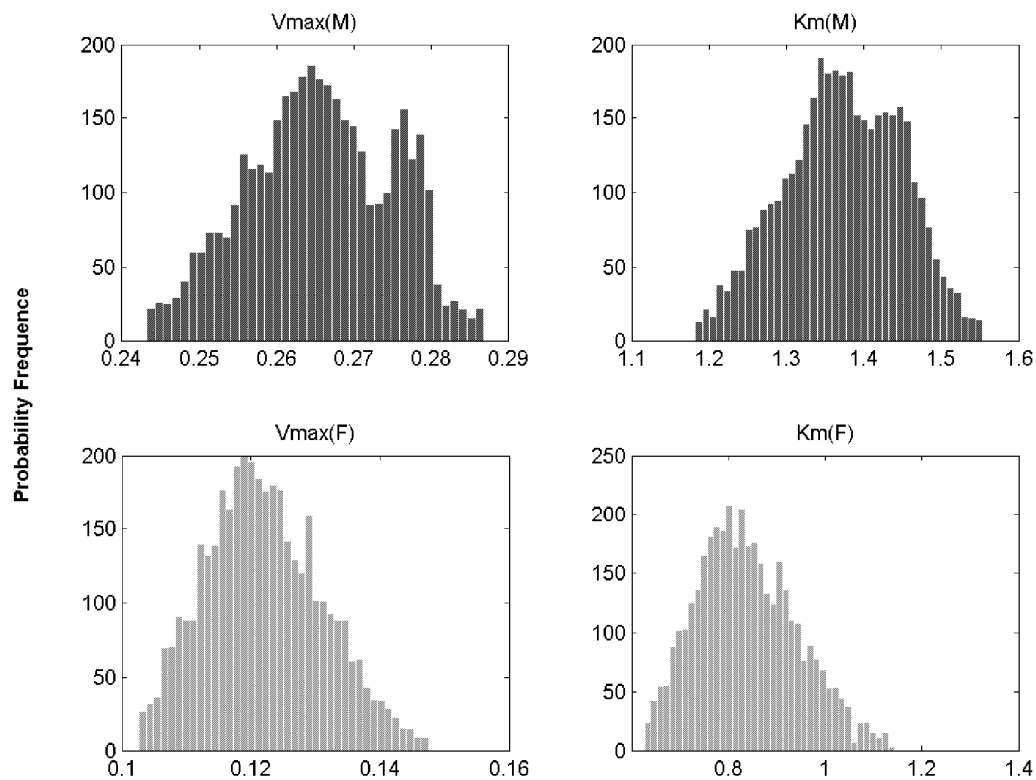
C3: Probability frequency of chloroprene oxidative metabolism parameters

Figure S3. Representative comparison of uniform prior and posterior distributions for human (pooled mixed gender) liver microsomal metabolism parameters



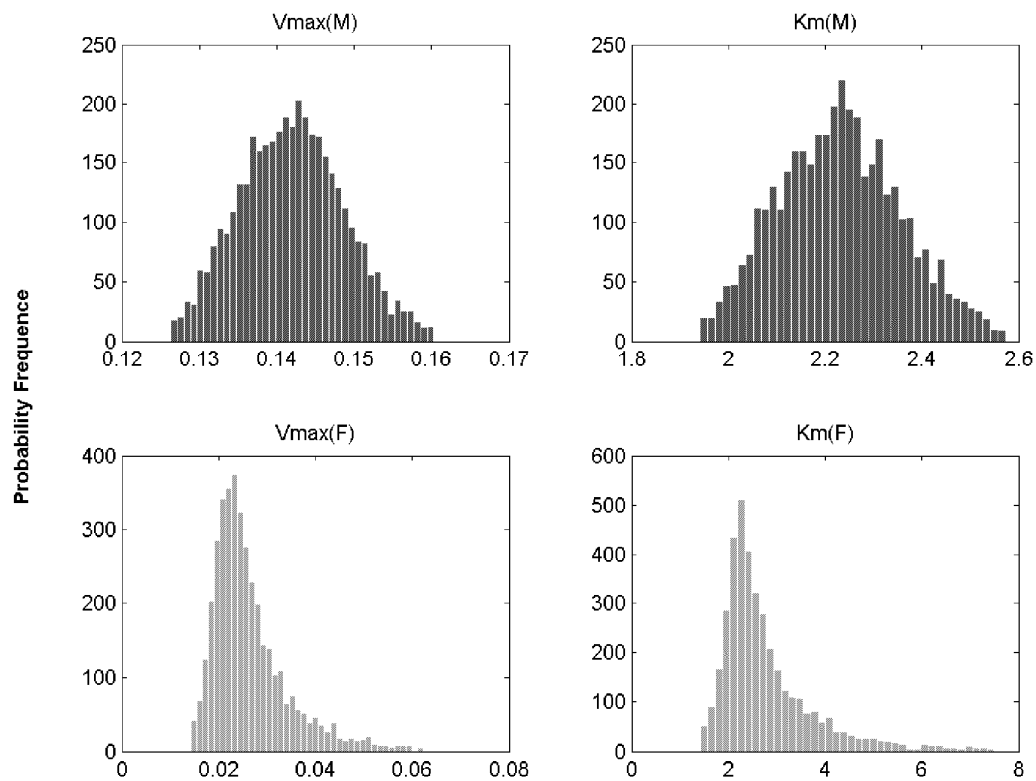
Note: Vmax ($\mu\text{mol/hr/mg}$ microsomal protein) or Km ($\mu\text{mol/L}$) posterior frequency counts (per 4000 simulations).

Figure S4. Probability frequency of chloroprene oxidative metabolism parameters in male (M) and female (F) B6C3F1 mouse liver microsomes



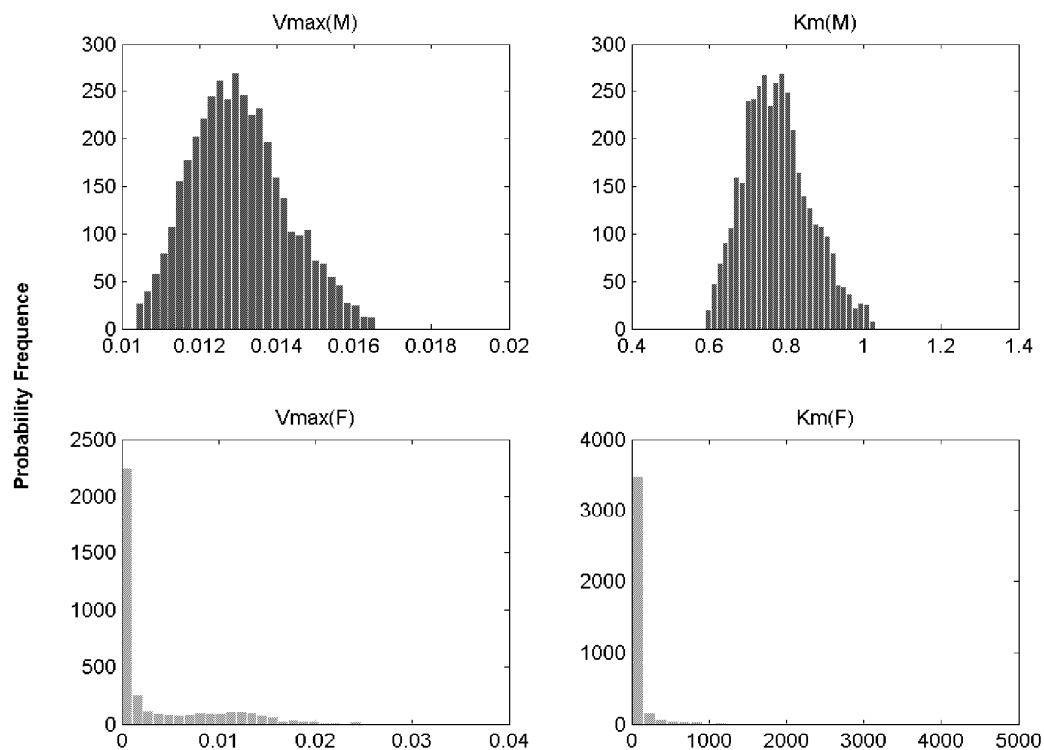
Note: Vmax ($\mu\text{mol/hr/mg}$ microsomal protein) or Km ($\mu\text{mol/L}$) posterior frequency counts (per 4000 simulations).

Figure S5. Probability frequency of chloroprene oxidative metabolism parameters in male (M) and female (F) B6C3F1 mouse lung microsomes

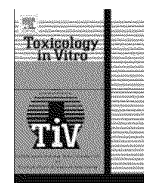


Note: Vmax ($\mu\text{mol/hr/mg}$ microsomal protein) or Km ($\mu\text{mol/L}$) posterior frequency counts (per 4000 simulations).

Figure S6. Probability frequency of chloroprene oxidative metabolism parameters in male (M) and female (F) B6C3F1 mouse kidney microsomes



Note: Vmax ($\mu\text{mol/hr/mg}$ microsomal protein) or Km ($\mu\text{mol/L}$) posterior frequency counts (per 4000 simulations).



Kinetic modeling of b-chloroprene metabolism: Probabilistic in vitro–in vivo extrapolation of metabolism in the lung, liver and kidneys of mice, rats and humans

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b-Chloroprene (chloroprene) is carcinogenic in inhalation bioassays with B6C3F1 mice and Fischer rats, but the potential effects in humans have not been adequately characterized. In order to provide a better basis for evaluating chloroprene exposures and potential effects in humans, we have explored species and tissue differences in chloroprene metabolism. This study implemented an in vitro–in vivo extrapolation (IVIVE) approach to parameterize a physiologically based pharmacokinetic (PBPK) model for chloroprene and evaluate the influence of species and gender differences in metabolism on target tissue dosimetry. Chloroprene metabolism was determined in vitro using liver, lung and kidney microsomes from male or female mice, rats, and humans. A two compartment PK model was used to estimate metabolism parameters for chloroprene in an in vitro closed vial system, which were then extrapolated to the whole body PBPK model. Two different strategies were used to estimate parameters for the oxidative metabolism of chloroprene: a deterministic point-estimation using the Nelder-Mead nonlinear optimization algorithm and probabilistic Bayesian analysis using the Markov Chain Monte Carlo technique. Target tissue dosimetry (average amount of chloroprene metabolized in lung per day) was simulated with the PBPK model using the in vitro-based metabolism parameters. The model-predicted target tissue dosimetry, as a surrogate for a risk estimate, was similar between the two approaches; however, the latter approach provided a measure of uncertainty in the metabolism parameters and the opportunity to evaluate the impact of that uncertainty on predicted risk estimates.

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1. Introduction

b-Chloroprene (chloroprene, 2-chloro-1,3-butadiene, CAS 126–99–8) is a volatile colorless liquid used to manufacture polychloroprene, a synthetic rubber (Lynch, 2001a). Occupational exposure can occur during monomer synthesis, shipping, and polymerization processes, and inhalation is the only significant route of exposure (Lynch, 2001b). The health effects in humans have focused on the potential carcinogenicity of chloroprene in the liver, lung and lymphohematopoietic systems (reviewed by Bukowski, 2009). Although epidemiological findings do not support a substantial link between chloroprene exposure and increased cancer mortality (Marsh et al., 2007), it is still important to understand species differences.

Extensive animal studies have been performed to understand possible adverse health effects of chloroprene in humans including acute, sub-chronic, and chronic toxicity studies (Melnick and Sills, 2001; Valentine and Himmelstein, 2001; Pagan, 2007). The most toxicologically significant finding was chloroprene-induced

tumorigenicity in F344/N rats and B6C3F1 mice exposed to 680 ppm for 2 years (Melnick et al., 1996, 1999; NTP, 1998). Tumors in Fischer rats included the lung, oral cavity, thyroid gland, kidney, and mammary gland. Mouse tumors were in the lung, circulatory system, Harderian gland, forestomach, kidney, mammary gland, skin, mesentery, Zymbal gland, and liver. In contrast, no tumors occurred in Syrian hamsters and only a weak response in mammary tissue in female Wistar rats (Trochimowicz et al., 1998) indicating species and gender differences in tumorigenesis in rodents.

Chloroprene is oxidized by cytochrome P450 enzymes (Cottrell et al., 2001; Himmelstein et al., 2001b). One reactive intermediate formed is the epoxide (1-chloroethenyl) oxirane which was mutagenic in the Ames assay, but not clastogenic at cytotoxic concentrations in vitro (Himmelstein et al., 2001a). This epoxide also shows reactivity with DNA in vitro and is a potential cross-linking agent (Munter et al., 2002; Wadugu et al., 2010). The reactive metabolites of chloroprene are likely to contribute to the tumorigenicity of chloroprene seen in animal studies. Given the important role of metabolic activation for toxicity, it is important to understand chloroprene metabolism to assess its potential health effects. To this end, a physiologically based pharmacokinetic (PBPK) model

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was developed for chloroprene based on in vitro metabolism data. Previous PBPK models for chloroprene in male rodents and humans (Himmelstein et al., 2004a, b) suggested significant differences in chloroprene metabolism among liver and lung and among different species. Intrinsic clearance of chloroprene metabolism in hepatic microsomes was two fold higher in mouse compared to human, while clearance in lung microsomes was forty times higher in mouse than either rat or human. With the application of PBPK modeling, the species differences in metabolism (amount chloroprene metabolized per gram lung tissue) were shown to be the underlying mechanism for the difference in lung tumor incidence among different species (Himmelstein et al., 2004b).

Here we extend the chloroprene PBPK model using additional data for chloroprene metabolism from different species and genders. The models evaluated the role of metabolism differences in species- and sex-dependent tissue dose metrics (a potential marker for tumorigenesis). A key objective of this effort was to develop a probabilistic parameter estimation approach; so that the impact of uncertainty in the metabolic parameter estimates on risk predictions can be as illustrated in Fig. 1. While previous studies used deterministic approaches to estimate metabolism parameters, we estimated these parameter values by two different methods: deterministic point-estimation and a probabilistic (Bayesian) approach.

2. Materials and methods

2.1. In vitro microsomal experiments

2.1.1. Chemicals

b-Chloroprene (>99%) containing phenothiazine and N-nitrosodiphenylamine inhibitors was supplied by DuPont Performance Elastomers, LLC (LaPlace, LA). The inhibitors were removed as previously described (Himmelstein et al., 2001b). The purified chloroprene was stable at <ff70 ffC under nitrogen headspace atmosphere. For metabolism experiments, vapor concentrations were prepared by adding the liquid test substance to Tedlar[®] bags (SKC Inc., Eighty Four, Pennsylvania, USA) containing a known volume of room air. Further gas phase dilutions were made for calibration or exposure purposes. Gas tight syringes were used for the gas transfers.

2.1.2. Source of microsomes and cytosol

Fischer rat (F344/DuCrI) and mice (B6C3F1/CrI) were received from Charles River Laboratories, Inc., Raleigh, North Carolina. The species and strains were selected to match those used for inhalation toxicity testing by the National Toxicology Program (NTP, 1998). The animals were acclimated for at least 7 days prior to use. A total of 15 female rats and 50 female mice were used for preparation of the liver and lung microsomes. A total of 15 rats/sex and 30 female mice/sex were used for preparation of kidney microsomes. Human

kidney microsomes were purchased from Xenotech (H0610.R, Lot No. 0810236, Lenexa, Kansas, USA). Microsomes were prepared by differential centrifugation and pooled as described by Himmelstein et al. (2004a). The use of pooled tissue microsomes mitigates issues of inter-animal biological variability, yet supports the analysis on species and gender differences. Further details on the microsomal preparation are given in the Supplement data A.1

2.1.3. Microsomal oxidation of chloroprene

The time course of total chloroprene disappearance was measured in three tissues: liver and lung microsomes for female rodent; kidney microsomes of rodents for both genders and human kidney microsomes. Data on the (1-chloroethenyl) oxirane formation was not collected in the current experiments because of the focus on total chloroprene metabolism as a dosimetric for dose–response modeling (Himmelstein et al., 2004b). After pre-incubation (37 ffC for 5 min), an equal volume of vial headspace was removed from the vial and replaced with known concentrations of chloroprene vapor. The vial was equilibrated for approximately 10 min and reactions were started by the addition of microsomal protein and NADP⁺ (0.53 mM). Microsomal protein concentrations were established from previous work (liver and lung) or experimentally for kidney microsomes. Definitive experiments used protein concentrations that ranged from 1–3 mg/mL. Control incubations were performed without NADP⁺ or with NADP⁺ and heat-inactivated microsomes. Samples (200 fL) were injected on the GC using a robotic x-y-z programmable multipurpose sampler (MPS2, Gerstel US, Baltimore, Maryland, USA) and were analyzed at 12 min intervals for up to 1 h.

2.2. In Vitro Kinetic Model Description

A 2-compartment PK model modified from Himmelstein et al. (2004a) was used to describe the time-concentration measurements of chloroprene in the headspace in the closed vial system. The microsomal oxidation of chloroprene in tissues (liver, lung and kidney) was by saturable kinetics, with the exception of rat and human lung where a first-order process was used. In addition to microsomal metabolism, the current model included the loss of chloroprene from the headspace to describe the decline of headspace concentration of chloroprene observed in the control dataset. The background loss rates were modeled as a first order process. Estimates of the first order background loss rates were based on eight sets of control data (the complete female data set plus the male kidney dataset). The in vitro experimental background loss rate was assumed to be independent of gender, tissue, and dose. The same PK model was used to estimate the background loss rate by setting the parameter values for the microsomal process to zero. To estimate the gender-specific variability of the kinetic parameters, male tissue

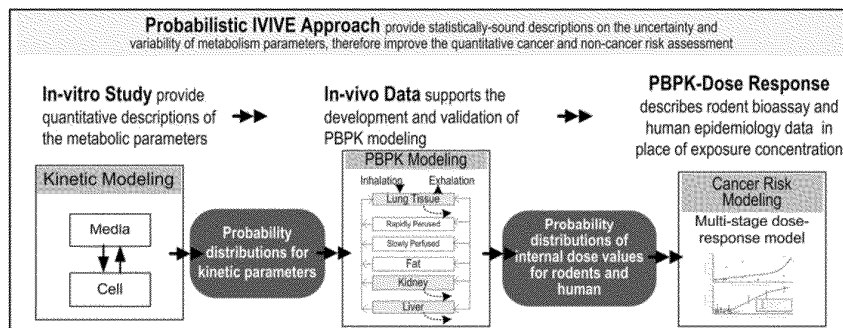


Fig. 1. Illustration of probabilistic approach.

data from Himmelstein et al. (2004a) were re-evaluated using the updated PK model. For a more detailed description of the male dataset and the 2-compartment model, see Himmelstein et al. (2004a).

2.3. Kinetic parameter deterministic (point) estimation

All model parameters were optimized with ACSL-Optimize (version 11.8.4, AEGIS, Technologies Group, Inc, Huntsville, Alabama, USA), using the Nelder-Mead method with a relative error minimization-based, log-likelihood function.

2.4. Kinetic Parameter Probabilistic (Bayesian) Analysis

2.4.1. Two-level hierarchical Bayesian model for rat and mice

A two-level hierarchical Bayesian model was used to estimate the gender-variability of the in vitro metabolic parameters. This approach was hierarchical in the sense that the uncertain population level (species) parameters at the top level define the variability of the lower-level (gender) parameter values. Inter-gender variability for a given microsomal activity parameter (in log-scale) was described by a normal distribution with population mean M and standard deviation S . The prior distribution of M was uniform (Table 1). The same log-uniform distributions were used for all model parameters (V_{max} , k_m and V_{max}/k_m) for all animal species, tissues, and doses. The log-uniform distribution [ff10, 5] was broad enough to encompass the actual distributions of the metabolic parameters. The initial mean values were determined from the point estimation results in Himmelstein et al. (2004a), and two preliminary MCMC analyses. Before a fixed log-uniform distribution [ff10, 5] was selected, two uniform distributions were tested for microsomal activity parameters; one [1e-8, 500] (natural scale); and the other [ff10, 10] (log-scale). All three priors produced the identical posterior results given the same variability and error model. The log-uniform [ff10, 5] was chosen to reduce the computational sampling time.

Prior descriptions of gender-specific variability (S) were lognormal [0.3, 5]. Because the MCMC parameters were sampled in log-space, the estimated gender-specific variability was an equivalent description to the coefficient of variation. One additional distribution, lognormal [0.3, 1], was tested in the preliminary analysis. Given the same prior conditions on other parameters, the posterior results obtained from the alternative priors for gender-specific variability were very comparable. The broader prior (lognormal [0.3, 5]) was selected to avoid over-constraining the posterior parameters. Computational procedures for the MCMC analysis are provided in Supplemental data A.2.

2.4.2. Population-only Bayesian model for human

Gender-specific microsomal activity data were not available for human tissues. A single-level MCMC simulation was performed using the prior distributions and likelihood functions from the 2-level hierarchical model. Estimation of population-only posterior distributions reflected the combined uncertainty and variability of model parameters calculated using the mixed gender microsomal human data.

2.4.3. MCMC computation process

Nine MCMC analyses were performed for this study (control dataset for background loss rate; liver, lung, and kidney for rat and mouse, and liver and lung for human). The human kidney microsomal metabolism data was not modeled because of the failure to observe experimentally measurable chloroprene uptake. Three MCMC chains were run for each analysis. A minimum of 200,000 iterations were performed for each chain. The first 100,000 iterations initialized the Monte Carlo chain ('burn-in' period) and the remaining 100,000 iterations were used for convergence testing and data analysis.

Table 1

Prior distributions for in vitro chloroprene metabolism parameters.

Parameter application	V_{max} , k_m , V_{max}/k_m ^a	
	Distribution	Truncation
Population (exp(M))	Uniform	[4.5e-5, 150]
Gender variability (S)	Lognormal (0.3, 5)	[0.01, 10]
Individual (exp(m)) ^b	Exp(Normal (M, S))	[2e-9, 2e4]

M – mean, exp(M) – exponential of mean, S – standard deviation.

^a Units: V_{max} (l mol/h/mg), k_m (l mol/L), V_{max}/k_m (L/hr/g protein).

^b Individual level parameter refers to gender-specific metabolic parameters in the 2-compartment in vitro PK model.

The MCMC analysis of background loss rate was executed first, prior to the other eight MCMC analyses. The derived posterior distribution of background loss rate was used as a fixed input for the MCMC analyses of chloroprene oxidation data for various tissues and species to account for the background loss of chloroprene in the headspace (in addition to removal of chloroprene during headspace sample extraction).

The method of Brooks and Gelman (1998) was used to diagnose the convergence of MCMC chains. Three MCMC chains were run for each analysis. Once the MCMC chains converged to a stationary distribution, the "converged" parts of the chains were considered representative samples from the posterior distributions (corrected scale reduction factor (CSRF) <1.2). After the chains converged, 4000 sets of the parameters were randomly sampled to represent the posterior distributions. Presentation of the results included probability frequencies, mean (exp(m)) and standard deviation (std(exp(m))) estimates of the 50th percentile central tendencies, and time course plots of chloroprene headspace concentrations with model estimates for a distribution of 50 simulated samples.

An example of the model code for one of the MCMC analyses is provided in the Supplementary data. This MCMC analysis was performed using acslX by The AEGIS Technologies Group, Inc.

2.5. In vitro–in vivo extrapolation of chloroprene metabolic Constants

2.5.1. PBPK model structure

A chloroprene PBPK model was first developed by Himmelstein et al. (2004b) to describe inhalation exposure of this chemical in mice and rats. The current model structure was adapted from this multispecies PBPK model with the addition of a kidney compartment. It is a flow-limited PBPK model with six tissue compartment: lung, liver, kidney, fat, slowly and rapidly perfused tissues. As in the original model, metabolism of chloroprene was included in the liver and lung mediated by cytochrome-P450 enzymes. This oxidative pathway was saturable process except in the lung in rats and humans. For these two cases, it was a first-order process. Metabolism of chloroprene in the kidney was via a P450 mediated saturable pathway for rats, mice, and humans.

Most of the physiological and biochemical parameters used in the current PBPK model were from the original model, with some modifications. Physiological parameter values were adapted from Brown et al. (1997). Tissue-to-blood partition coefficients were calculated using the means of the experimental tissue-to-air partition values in Himmelstein et al. (2004b). The partition coefficients for rapidly perfused and slowly perfused tissue compartments were the same as those reported for the kidney and muscle, respectively. In vitro-derived gender-specific metabolism parameters were used to estimate chloroprene metabolism in liver, lung and kidney. Scaling of these in vitro parameters to the corresponding in vivo parameters followed the same steps as described in Himmelstein et al. (2004b). It was assumed that there is no-gender difference in the microsomal protein contents among different species, the

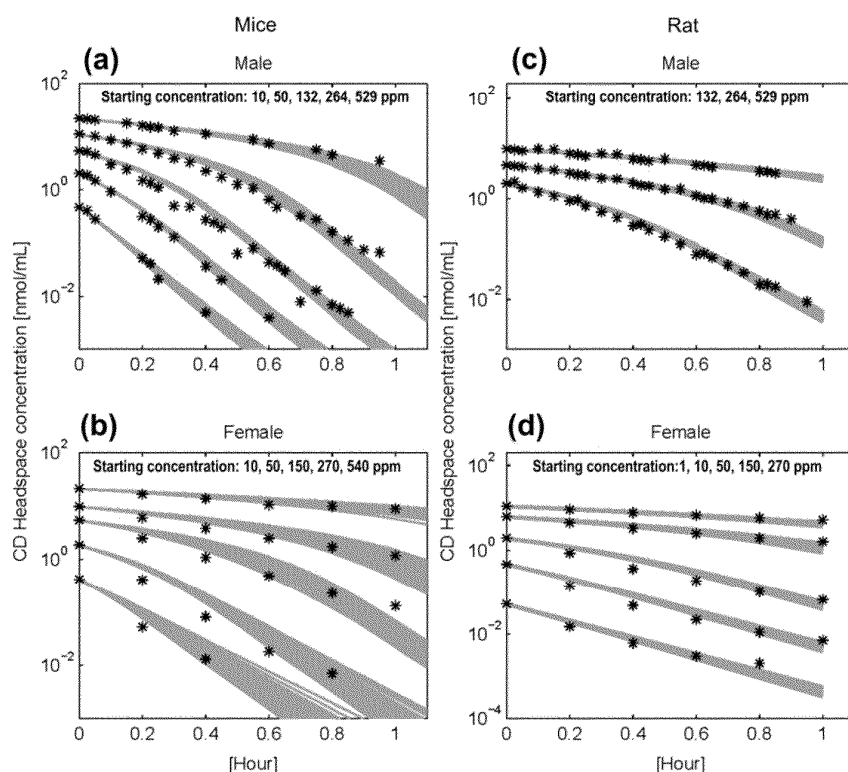


Fig. 2. Distributions of chloroprene oxidative metabolism time-course predictions versus experiment data (symbols) in liver microsomes. Chloroprene headspace concentrations were collected for various starting headspace concentrations. Simulated time course data (lines) were based on posterior distribution for parameter values reported in Table 5. Simulations represent 250 sets of model parameters randomly drawn from the posterior distributions.

values of which for the liver were 35, 49, and 56.9 mg protein/g liver for mice, rats, and humans, respectively (Himmelstein et al., 2004b). For lung and kidney microsomes, 23 and 11.5 mg protein/g was used for all animal species (Himmelstein et al., 2004b).

To quantitatively compare the effect of gender difference of the metabolism parameters on the dose–response analysis, we calculated the gender-specific internal tissue dose, i.e., the average amount metabolized per day per gram of lung (AMPLU) using the PBPK model. The dose metric AMPLU was selected as a surrogate for the target tissues dose based on the mode of action and cancer dose–response analysis (Himmelstein et al., 2004b). The PBPK-derived AMPLU values were calculated using both point- and probability-based metabolic parameters. For the deterministic approach, the updated liver, lung and kidney metabolism parameter estimates from the Nelder–Mead algorithm were scaled allometrically and used in the PBPK model to calculate gender-specific AMPLU values for mice, rats and humans. For the probabilistic approach, the Monte Carlo technique was employed to calculate the distribution of the gender- and species-specific AMPLUs by sampling and scaling from the posterior distributions of the metabolism parameters for liver, lung and kidney estimated during the MCMC analysis of in vitro metabolisms parameters.

3. Results

3.1. Microsomal oxidation of chloroprene in liver, lung and kidney tissues

The purpose of this study was to investigate the species, gender, and tissue differences in chloroprene metabolism. To this

end, gender-specific microsomal oxidation in liver, lung and kidney were measured in rat, mice, and human using pooled microsomal samples. Estimates of metabolic rate parameters were based on two-compartment modeling of a family of time course curves for each experimental factor (species, sex, and tissue type). The gender- and species-specific metabolic clearance of chloroprene in microsomes is shown by the data points in Fig. 2–5, which represent the disappearance of chloroprene in the head space of a closed vial system for liver and lung in female rats and mice; kidney microsomes of rodents of both genders, and human kidney microsomes. These data were used to estimate in vitro metabolic parameters for chloroprene as described below.

3.2. Parameter estimation using the deterministic approach

The point estimate of the background loss rate constant was 1.41 L/hr/g. The point estimation results for the microsomal oxidation parameters with background loss rate are presented in Table 3. Oxidation parameter estimates without background loss rate correction were also optimized for comparison purposes. Even with the background loss rate, microsomal oxidation was observed in most of the tissues. In some tissues it was possible to see an impact of considering background loss; for example the estimated intrinsic clearance dropped from 1.3 to 0.9 L/hr/g in the male rat lung microsomal incubations. The greatest impact was for the female mouse kidney where the intrinsic clearance decreased from 0.83 to 0.024 L/hr/g. The comparison of chloroprene headspace measurements and model predictions simulated using point estimates of the model parameters are in the Supplemental data.

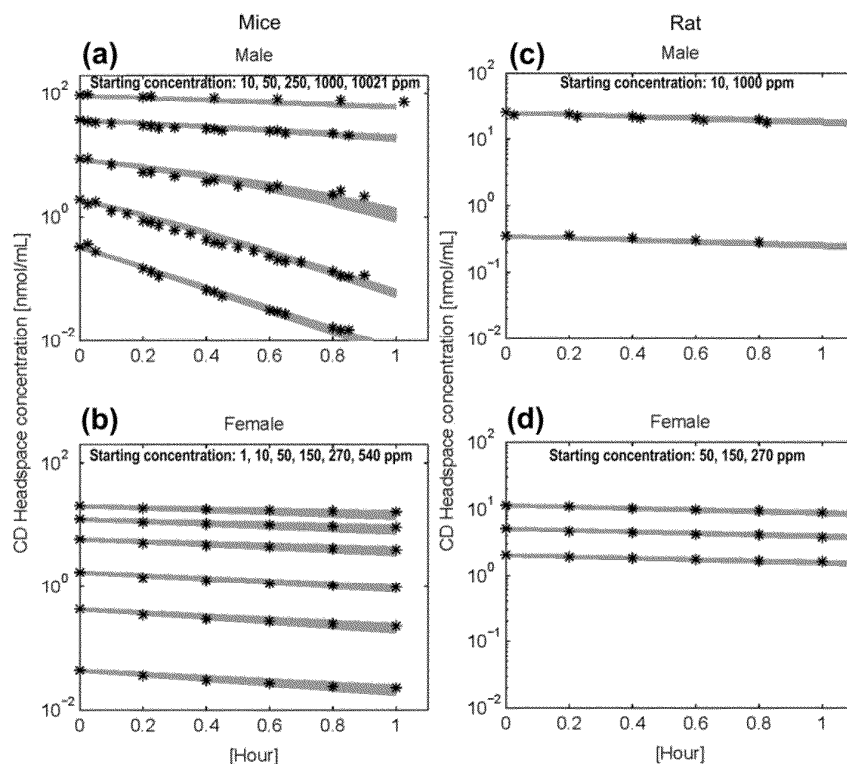


Fig. 3. Distributions of chloroprene oxidative metabolism time-course predictions versus experiment data (symbols) in lung microsomes. Chloroprene headspace concentrations were collected for various starting headspace concentrations. Simulated time course data (lines) were based on posterior distributions for parameter values reported in Table 5. Simulations represent 250 sets of model parameters randomly drawn from the posterior distributions.

3.3. Parameter estimation using the probabilistic approach

Intrinsic clearance (V_{\max}/k_m) for background loss was calculated from the geometric mean values for V_{\max} and k_m ; the resulting 95th, 50th and 5th percentile of the posterior distribution were 1.5, 1.4, and 1.3 L/hr/g, respectively. The convergence of the MCMC results was verified based on CSRF values (see Section 2) which were below 1.1 for all the parameters.

Estimates of the enzyme-mediated metabolic constants are presented in Table 4. The means of the posterior distributions of the metabolic parameters showed excellent agreement with those of the point estimates (Table 3). The point estimates were typically within one standard deviation of the posterior mean values (Table 4). One exception was the intrinsic clearance for the female mouse kidney where the Bayesian estimate (0.25 L/hr/g) was 10-fold higher than the point estimate (0.024 L/hr/g). The uncertainties in the model parameters were significantly reduced from the prior distributions, as demonstrated by the narrower posterior distributions (Table 4). For all species, the metabolic capacity in microsomes was the highest in the liver, followed by the lung or kidney (Tables 3 and 4). Gender differences were observed in all tissues examined (Kruskal–Wallis ANOVA, $p < 0.0001$, Table 4). The intrinsic clearance (V_{\max}/k_m) determined in liver microsomes was higher in males than in females both for rats and mice. Species differences in the tissue intrinsic clearance rate were also observed. Higher clearance was estimated in the lung than the kidney for mice; but this was reversed for rats (Tables 3 and 4). Figs. 2–5 present the distributions of the rate of chloroprene metabolism simulated with metabolic constants randomly drawn from their posterior distributions. The width of the band showing 250 randomly selected simulations reflects the impact of the uncertainty of the metabolic parameters on the

distribution of the model output, i.e., chloroprene concentration in this case. The point estimation and Bayesian method both provided good agreement with the in vitro experiential observations.

3.4. Internal dose calculations

AMPLU was calculated using the PBPK model with the updated metabolic rate constants from the in vitro studies (Table 2). PBPK model-predicted AMPLUs based on the metabolism parameters scaled from both the deterministic and probabilistic approaches were compared (Table 5). The AMPLU values were comparable between the two methods, the means estimated with the point estimation fall within the probabilistic distribution of AMPLUs from the MCMC analysis. Results from both approaches showed that the total metabolism per gram lung was greatest in mice followed by rats and humans. Both approaches indicated that AMPLU was linear for the rats and human over the selected bioassay concentrations (12.8, 32, or 80 ppm) but indicated saturation for the mouse, consistent with the previous dose–response analysis for male rats and mice (Himmelstein et al., 2004b). A gender difference in the AMPLU estimates was observed for both rats and mice; however, they were more significant in mice (male mouse AMPLU was 4–5 times higher than the values estimated for female mouse).

4. Discussion

One of the major challenges in using PBPK models in risk assessment is the issue of uncertainty and variability in model predictions. Until recently, most of the PBPK models have been developed based on point estimates for the physiological and chemical-specific

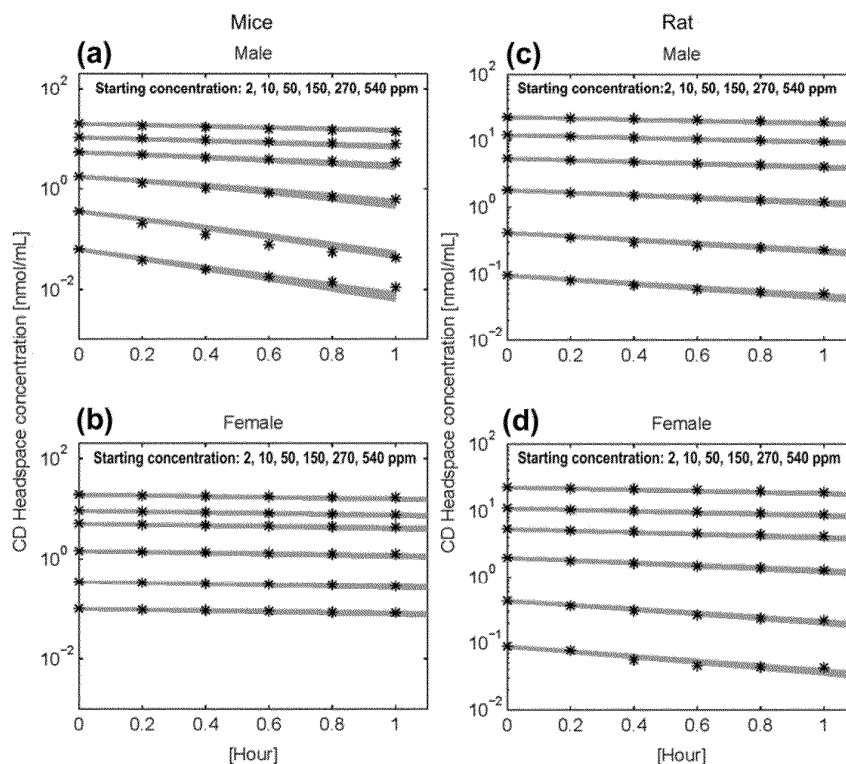


Fig. 4. Distributions of chloroprene oxidative metabolism time-course predictions versus experiment data (symbols) in kidney microsomes. Chloroprene headspace concentrations were collected using various starting headspace concentrations. Simulated time course data (lines) were based on posterior distributions for parameter values reported in Table 5. Simulations represent 250 sets of model parameters randomly drawn from the posterior distributions.

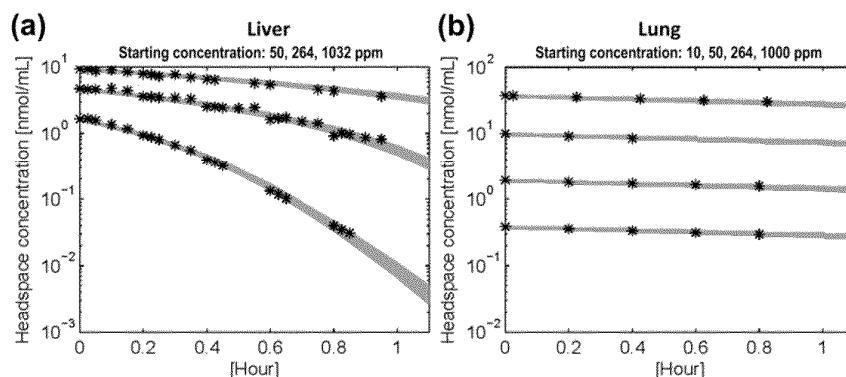


Fig. 5. Distribution of chloroprene oxidative metabolism time course predictions in liver and kidney microsomes for human. Symbols represent measured chloroprene headspace concentrations. Model simulations (lines) were based on posterior distributions of parameter values as reported in Table 5. Simulations represent 250 sets of model parameters randomly drawn from the posterior distributions.

parameter values, and consequently have predicted a single kinetic behavior of the chemical in the body. However, differing degrees of uncertainty are expected both for physiological and chemical-specific parameter estimates, especially metabolism constants, which will result in a corresponding range of model predictions for the dose metric of interest. For the purposes of this investigation – evaluating the risks of chloroprene exposure – the issue to be addressed is the impact of true uncertainty (uncertainty regarding the central estimate of a particular metabolic parameter in a given species, gender, and tissue) on risk estimates (target tissue dosimetry). No attempt was made to characterize population variability in either

the rodent or the human. Risk assessments for chloroprene have been based on summarized outcomes (incidence) in groups of animal or workers, and the goal of these assessments has been to provide a central or upper-bound estimate of the risk to an average individual. Therefore, our focus on true uncertainty is appropriate. Although the National Academy of Science (NAS, 2009) has recently recommended greater consideration of human variability in risk assessment, this would require a much more ambitious experimental study involving a large number of human tissues.

In an effort to characterize the impact of metabolic parameter uncertainty on a risk assessment for chloroprene, we applied a

Table 2
In vivo PBPK model parameters.

Parameter	Mouse		Fischer rat		Human
Body weight (kg)	0.03		0.25		70
Ventilation (L/h/kg ^{0.75})	30		21		16
Cardiac output (L/h/kg ^{0.75})	30		18		16.2
Tissue volumes (%BW)					
Liver	5.5%		4.0%		2.6%
Kidney	1.4%		1.0%		1.0%
Fat	5.0%		7.0%		21.4%
Rapidly perfused	1.4%		3.5%		9.1%
Slowly perfused	77.0%		75.0%		56.1%
Lung	0.7%		0.5%		0.8%
Blood flows (%CO ^a)					
Liver	16.1%		18.3%		22.7%
Kidney	10.0%		14.0%		14.0%
Fat	7.0%		7.0%		5.2%
Rapid perfused	51.9%		45.7%		33.2%
Slow perfused	15.0%		15.0%		24.9%
Partition coefficients					
Blood:air	7.83		7.35		4.54
Liver: blood	1.25		1.57		1.44
Kidney: blood	1.76		2.27		2.64
Fat: blood	17.29		16.87		28.38
Rapid perfused: blood	1.76		2.27		2.64
Slow perfused: blood	0.58		0.60		0.99
Lung: blood	2.38		1.84		2.92
Metabolism	Male	Female	Male	Female	Mixed
V _{max} C, Lung	0.60 ± 0.03 ^b	0.11 ± 0.05			
k _m , Lung	0.20 ± 0.01	0.25 ± 0.13			
KF, Lung			0.15 ± 0.03	0.16 ± 0.02	0.05 ± 0.04
V _{max} C, Liver	18.54 ± 0.75	8.88 ± 0.84	9.48 ± 0.25	9.37 ± 0.52	20.4 ± 0.36
k _m , Liver	0.12 ± 0.008	0.08 ± 0.01	0.05 ± 0.003	0.09 ± 0.006	0.04 ± 0.001
V _{max} C, Kidney	0.078 ± 0.007	0.03 ± 0.05	0.018 ± 0.002	0.018 ± 0.002	
k _m , Kidney	0.068 ± 0.008	9.59 ± 44	0.067 ± 0.009	0.053 ± 0.007	

^a CO – cardiac output.

^b Mean ± SD from Markov Chain Monte Carlo posterior distribution.

probabilistic Bayesian approach to estimate metabolism parameters from in vitro data in which distributions, rather than point estimates, of estimated parameters (posterior distributions) were generated reflecting the uncertainty in the metabolism parameters. To our knowledge this study marks the first time a probabilistic approach has been employed to estimate a distribution for PBPK model parameters from in vitro metabolism studies. We also compared a deterministic approach, nonlinear optimization, with the probabilistic Bayesian approach. For the deterministic approach, the parameters were optimized to provide the maxima likelihoods between the prediction and the data determined in vitro. In the Bayesian approach, relatively non-informative distributions were used as priors (e.g., uniform distributions) so that the posterior distributions of the parameters would be estimated primarily on the likelihood of the parameters given the data. Thus, the metabolic parameters were estimated based on data-likelihood regardless of the methods used for parameter optimization. Our results show that the in vitro metabolism parameters obtained from the two different approaches are consistent: the point estimates for the parameters from the deterministic method are within the posterior distributions obtained from the MCMC analysis. The parallel parameter estimation using both deterministic and the probabilistic methods provided us an opportunity to evaluate the uncertainty in the resulting model parameters while still being able to compare the results from the current modeling to those from the previous modeling effort in which the parameters were optimized by a deterministic approach.

The estimated uncertainty in the in vitro metabolism of chloroprene was higher in female than male rodents. The uncertainty in the data was likely smaller in the males due to the greater amount

of data available. In the case of kidney, the same numbers of measurement were collected for both genders. In this case, the larger uncertainty observed with the in vitro-data for female mice may result from the lower rates of chloroprene metabolism in kidney microsomes from female mice. The lower clearance of chloroprene by kidney makes it more difficult to distinguish metabolism from background vial loss. This lower rate of kidney metabolism in the female mouse is not unexpected since chloroprene inhalation caused kidney toxicity in male, but not female, mice (Melnick et al., 1999).

Our probabilistic and deterministic approaches resulted in similar estimates for parameter values. However, the use of a probabilistic approach allowed us to evaluate the uncertainty in the estimates of dose metric, in this case AMPLU, as a result of uncertainty in the in vitro data. To accomplish this, the PBPK model and the posteriors of metabolic PBPK model parameters (liver, lung, and kidney) from the in vitro MCMC analysis were used to simulate the distribution of AMPLU via Monte Carlo techniques. The resulting variance of the AMPLU distribution, represented by the coefficient of variation (CV), increased with exposure concentration in the case of mice; but not for rats and humans. For mice, the CV was doubled when exposure concentration was increased from 12.8 to 80 ppm in both genders (3.8%–7.6% for male and 11%–24% for female). For rats, the CV was about 20% for females for the three concentrations tested, which were slightly higher than male rats (about 14%). This difference likely reflects the fact that lung metabolism in mice was described as a saturable (non-linear) pathway while a linear pathway was used for rat and human. For humans, the AMPLU values were the lowest among the species, and the population variation of AMPLU attributable to the uncertainty in the metabolism

Table 3
Point estimate values for the microsomal oxidation of chloroprene.

Species	Sex	Tissue	Metabolic parameters ^{a,b}		
			V _{max}	k _m	V _{max} /k _m
B6C3F1 mouse	Male	Liver	0.26	1.36	186
		Lung	0.13	2.0	64
		Kidney	0.01	0.5	20
	Female	Liver	0.09	0.53	174
		Lung	0.025	2.78	8.9
		Kidney	0.00004	1.7	0.024
F344 rat	Male	Liver	0.077	0.56	139
		Lung			0.9
		Kidney	0.0027	0.92	3
	Female	Liver	0.068	0.82	82
		Lung			1.2
		Kidney	0.00177	0.37	4.7
Human	Mixed	Liver	0.054	0.45	120
		Lung			0.9

^a Obtained by ACSL Optimization and includes correction for background loss of chloroprene during the incubation.
^b Units: V_{max} (l mol/h/mg); k_m (l mol/L); V_{max}/k_m (L/hr/g).

parameters was most significant (over 60%). The variation of the intrinsic clearance for the human lung was also the highest among tissues and species.

Overall, we used MCMC analysis as a probabilistic parameter optimization tool to provide estimates of metabolic parameter distributions for use in a PBPK model. This approach is consistent with the EPA's efforts to develop guidelines for probabilistic risk assessment. In the recent EPA "Risk Assessment Guidance for Superfund", it states "... methods used to quantify uncertainty in the model inputs are based on statistical principles such as sampling distributions (Monte Carlo analysis) or Bayesian approaches". In a Monte Carlo probability analysis (Buur et al., 2006; David et al., 2006; Thomas et al., 1996), the parameter variability was estimated by 'assigning' distributions around point estimates obtained from

literature reviews or in vitro data. Our approach used Bayesian techniques to generate the probabilistic distributions of model parameters based on statistical procedure and reflected the prior knowledge (when available) and the data from new experimental studies. It should be noted, however, that the posteriors from Bayesian analyses are calibrated to a particular data set, consideration must be given as to whether the subject populations in the data sets represent the population(s) of interest. For example, one would expect low variability of the kinetic parameters derived from animal experiments since the laboratory animals are more homogeneous. However, tissues in a human study may be less representative of the general population due to human heterogeneity. If there is interest in accounting for variability in the risk assessment, traditional Monte Carlo simulation can be performed where some of the Bayesian-based posterior distributions are replaced with distributions considered more representative of the population of interest (such as the defined population variability of CYP-mediate metabolism based on CYP polymorphisms and abundance (Lipscomb et al., 2004)).

In summary, this study presents a novel probabilistic approach to integrate in vitro metabolism data with physiologically based pharmacokinetic (PBPK) modeling. To achieve this goal, first, gender-specific in vitro microsomal data were collected in liver, lung and kidney for mice, rats, and humans. Second, gender- and tissue-specific metabolism parameters were estimated using a compartmental pharmacokinetic (PK) model and Bayesian analysis. Central estimation of the posterior distributions of parameters from the Bayesian analysis were compared to parameters obtained using a traditional optimization method to provide confidence in the values obtained. Third, the role of metabolism differences in species- and sex-dependent tissue dose metrics were investigated by running the PBPK model with the posterior parameter distributions. Our results show that in vitro-derived metabolism rate constant distributions can be linked in PBPK models to evaluate the role of metabolism differences in species- and sex-dependent tissue dose metrics, further to evaluate the resulting uncertainty in risk estimates of chloroprene.

Table 4
Probability analysis of microsomal oxidation parameters for chloroprene.

Species	Sex	Tissue	Metabolic constants ^a					
			V _{max} ^b		k _m ^b		V _{max} /k _m ^{c,d,e}	
			Mean	SD	Mean	SD	Mean	SD ^d
B6C3F1 Mouse	Male	Liver	0.26	0.01	1.34	0.08	194.7	5.50
		Lung	0.14	0.01	2.22	0.14	63.7	1.00
		Kidney	0.01	0.001	0.77	0.09	16.8	0.70
	Female	Liver	0.13	0.01	0.88	0.14	144.5	11.80
		Lung	0.03	0.01	2.82	1.51	9.7	1.10
		Kidney	0.004	0.01	176.11	922.87	0.25	0.26
F344 Rat	Male	Liver	0.10	0.003	0.56	0.03	138	3.65
		Lung					1.28	0.25
		Kidney	0.003	0.0003	0.76	0.11	3.3	0.18
	Female	Liver	0.09	0.01	0.56	0.03	78.6	1.75
		Lung					0.96	0.22
		Kidney	0.003	0.0003	0.60	0.08	4.2	0.16
Human	Mixed	Liver	0.05	0.001	0.45	0.01	122.2	2.20
		Lung					0.32	0.2
		Kidney						ND ^f

^a Mean (exp(m)) and standard deviation SD (exp(s)) values obtained by Markov Chain Monte Carlo (MCMC) analysis and includes correction for background loss of chloroprene during the incubation.
^b V_{max} (l mol/h/mg); k_m (l mol/L).
^c V_{max}/k_m (L/hr/g) calculated as V_{max}/k_m*1000 mg/g (unit conversion).
^d Mean and SD V_{max}/k_m estimated directly via MCMC analysis.
^e ND – metabolism not detected.
^f Tissue-specific microsomal activities were significantly different between the gender for rat and mice (Kruskal–Wallis ANOVA (nonparametric), p < 0.0001) (Supplemental data C).

Table 5
Estimation of PBPK-derived internal dose in the lung using deterministic and probabilistic approaches.

Species	Exposure conc. (ppm)	Gender	Internal dose ^a					
			Deterministic Approach ^b		Probabilistic approach ^c			
					Mean	CV%	5%	50%
B6C3F1 Mouse	12.8	Male	4.15	4.17	2.2	4.02	4.17	4.31
		Female	0.74	0.79	10.9	0.65	0.79	0.92
	32	Male	6.66	6.86	3.3	6.57	6.94	7.26
		Female	1.19	1.29	16.5	0.99	1.25	1.67
	80	Male	8.56	8.99	4.4	8.49	9.07	9.76
		Female	1.58	1.53	24.1	1.19	1.64	2.51
F344 Rat	12.8	Male	0.19	0.24	17.5	0.17	0.24	0.31
		Female	0.23	0.26	14.2	0.20	0.26	0.33
	32	Male	0.47	0.60	18.7	0.41	0.60	0.77
		Female	0.56	0.67	14.3	0.51	0.67	0.83
	80	Male	1.18	1.54	18.4	1.02	1.57	1.96
		Female	1.42	1.69	14.5	1.29	1.68	2.11
Human	12.8	Mixed	0.1	0.04	64.5	0.01	0.04	0.11
	32	Mixed	0.25	0.09	67.1	0.02	0.08	0.28
	80	Mixed	0.64	0.23	64.0	0.04	0.20	0.65

^a Internal dose = average daily umole CD metabolized/g lung tissue.

^b Calculated based on point-estimates of parameters (Table 3).

^c Calculated based on posterior distributions of tissue-specific metabolism parameters (Table 4).

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tiv.2012.04.004>.

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Sent: Wed 10/14/2015 12:47:53 PM
Subject: Comments on the chloroprene assessment
[DuPont PostEPR Comments 21Jan2010 final.pdf](#)
[12.17.09 DPE Comments on Draft Chloroprene IRIS Review.pdf](#)

Hi Norm,

I have been talking to Allen and George about details of the chloroprene assessment and Allen found some written comments (attached). I told him this will be very useful for our future discussions.

Ines Pagan

DVM, Ph.D.

Toxicologist

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Dear Mr. Davis:

DuPont Performance Elastomers (DPE) thanks the USEPA for the opportunity to present its positions at the Chloroprene External Peer Review Panel (Panel) meeting held on January 6, 2010.

While we appreciate the opportunity to express our views as part of the written record, issues were raised during the public Peer Review Panel meeting to which we were not permitted to respond. As described in our prior written and oral comments, the goal of DuPont Performance Elastomers, in collaboration with our International Institute of Synthetic Rubber Producers partners, has been to identify, conduct and communicate research supporting development of a scientifically sound and complete risk assessment for chloroprene. Consequently, we are providing you, as the IRIS Chemical Manager for chloroprene, with additional comments on points discussed during the Panel meeting where we both agree and disagree with Panel Member statements. In principle, we agreed with several points discussed by the Panel, notably

- Oral RfD values cannot be calculated from the existing data and route to route extrapolation cannot be conducted, since no validated oral kinetic data are available.
- In considering the species and effect used in the derivation of the RfC, we support the position that the most sensitive and relevant endpoint for chloroprene should be selected prior to any dose adjustment in dose-response modeling to define the Point of Departure (POD).

- Consideration of the rat as the more appropriate species for cancer risk assessment, given the questionable relevance of the mouse bronchioalveolar tumors to humans and the similar kinetics of chloroprene metabolism in the rat and human, compared to the mouse.
- Including an upper bound cancer risk estimate based on exposure data described in the Marsh et al. (2007a, b) epidemiology study to compare with the unit risk estimates derived using either the mouse or the rat data.
- We agree and see value in the use of models for HEC derivation. Using a validated PBTK model for chloroprene, as will be reported in the results of the forthcoming IISRP studies, will provide a robust approach to quantitatively account for the differences in toxicokinetics between rodents and humans.
- The lack of consideration of the DeWoskin (2007) peer-reviewed manuscript in the Draft Review was noted.

There remain a number of topics where our understanding of the science differs from the verbal positions articulated by the Panel. The issues which we believe merit a more in-depth scientific review include:

Evaluation of Epidemiological Data

- **It is inappropriate for the USEPA and the Panel Members to reach weight-of-evidence conclusions without understanding the limitations of the Eastern European and Asian epidemiology studies compared to the strengths identified with the Marsh et al. (2007) US and Western European epidemiology studies when using the 2005 USEPA criteria for evaluation of epidemiology study quality.**

We believe it is inappropriate for the USEPA to give equal weighting to the Eastern European and Asian epidemiology studies (Bulbulyan et al., 1998, 1999; Li et al. 1989) as that accorded to the key study conducted by Marsh et al. (2007a,b). As noted by multiple commenters, the Marsh et al. study provided the most complete and robust study of chloroprene human cancer risk to date. Further, an independent review of chloroprene epidemiology published by Bukowski (2009) that directly applies USEPA guidelines for assigning weight-of-evidence to epidemiologic studies should be included in the Draft Toxicological Review.

With respect to the weight-of-evidence discussions, we believe that the positions articulated by Panel Members misinterpret the significance of the Marsh et al study (2007a, b) by not appreciating: a) the lack of monotonic dose response and statistical significance in the relative risks (RR) for lung and liver cancers; and b) that the apparent increase in RR for lung and liver cancer as a function of chloroprene exposure is misleading without considering the impact of the spuriously low cancer deficits in the baseline population. Dr. Marsh provided further information at the Panel meeting and in the DPE written comments to conclude that there is no association between chloroprene exposure and liver or lung cancer.

In addition, it is scientifically indefensible to selectively use results from the Marsh et al. (2007a,b) study to present alternate interpretations of potential excess cancer risk that deviate from the conclusions published by the study authors. Revising the conclusions of the original authors of a published peer-reviewed study demands a comprehensive and justifiable rationale for doing so. Such a rationale was not provided in either the Draft Toxicological Review nor by Panel Members.

Overall, we maintain that the conclusions of the Marsh et al. (2007a,b) study should prevail in a weight-of-evidence analysis that considers the limitations of previous epidemiological investigations; in brief, the overall weight-of-evidence does not support the conclusion that chloroprene is “likely to be carcinogenic to humans”.

- **One Panel Member indicated that it was not possible to evaluate fully exposure-response in the Marsh *et al.* cohort study because the study investigators "had not performed lagged analyses or analyses by age at onset".**

This statement is incorrect because extensive lagged analyses were conducted as reported in the Marsh et al. (2007) paper and the DPE comments. Further, “age at onset” is not a relevant metric for evaluation in a mortality study when mortality outcomes are determined from death certificates. Cancer mortality was analyzed by Marsh et al. (2007) using multiple time-related factors (age at risk, age at hire, time since first exposure, etc.). These rigorous lagged and other time-related analyses supported the authors’ overall conclusion that their study provided no evidence of an exposure-response association for chloroprene with lung or liver cancer.

- **One Panel Member commented that the "healthy worker survivor effect" (HWSE), may have biased the analyses reported in the Marsh et al. studies.**

Dr. Marsh provided a detailed overview of several alternative explanations for spuriously low baseline rates for lung and liver cancer observed in the Marsh et al. cohort study and concluded that the HWE cannot be the sole cause for the phenomenon. This conclusion would also apply to consideration of the HWSE for the same reasons.

Essentially, the potential for a HWSE as a possible explanation for non-monotonic trends in relative risks was considered through the evaluation of lagged analyses as discussed above. Lagged analyses¹ remove more recent exposure periods from all workers’ cumulative exposure estimates and would adjust for longer employment periods for workers in Marsh et al. study (Checkoway et al., 2004). Therefore, lagged exposure periods reduce bias in the relative risk estimates for higher levels of cumulative exposure that may result from the preferential retention of healthier workers in the workplace. As discussed above, lagged exposure analysis showed no evidence of increased risk for cancer outcomes and did not materially change the interpretations of results from unlagged analyses.

- **One Panel Member commented that the periodic physical exams routinely given to workers at the Louisville plant included in the Marsh *et al.* study would have removed from the pool of eligible study members those individuals who were too ill to continue working.**

Workers in the Marsh et al. cohort study were followed for mortality outcomes through 2000 (1999 for the Grenoble, France plant) including workers with short duration of employment. There is no

¹ The use of lagged analyses to adjust for HWSE has been described in several research papers (Arrighi and Hertz-Picciotto, 1994; Gilbert 1982; Hertz-Picciotto et al., 2000).

evidence from the Marsh et al. study that any eligible study members had been omitted from the study population due to this or any other reasons.

- **One Panel Member also alluded to some unpublished NIOSH documents pertaining to the Louisville plant that should be evaluated as part of the analysis of lung and liver cancer rates.**

Selective incorporation of unpublished, non peer-reviewed materials should not be permitted until the USEPA establishes the validity or relevance of such materials.

Determination of Mode of Action

- **One of the primary arguments proposed by USEPA in support of a genotoxic mode of action and discussed by multiple Panel Members was the structural and biological activity similarities between chloroprene and 1,3-butadiene. In actuality, the genotoxic attributes of the two compounds are dissimilar when viewed across tests in common.**

Information	Chloroprene	1,3-Butadiene
Chemical is unequivocally mutagenic in the Ames Test (+/-S9)	NO Conflicting evidence as 2 of 4 studies were negative. Freshly prepared compound was negative (Westphal et al., 1994) ²	YES Clearly positive in the base-pair substitution strain TA1535 +S9 (Madhousree et al., 2002)
Chemical induces point mutation in mammalian cell culture assays (+/-S9)	NO No mutation induction in V79 cells (+/-S9). Vinyl chloride, however, was mutagenic in this study (Drevon and Kuroki, 1979)	YES Weak but positive response at the TK gene in the mouse lymphoma assay (Sernau et al. 1986)
Chemical is genotoxic in standard <i>in vivo</i> genetic assays following inhalation exposure.	NO Target tissue toxicity observed but no increase in aberrations, SCEs or micronuclei in B6C3F1 mice up to 80 ppm (Shelby, 1990)	YES Dose related increases in chromosome aberrations, SCEs and micronuclei induced in B6C3F1 mice (Shelby, 1990)
G to C base substitution mutations in K-ras codon 61 are observed in mouse lung tumors	NO Excess of A to T transversion mutations (22/25 in codon 61) alleged responsible for increase in lung tumors (Sills et al., 1999).	YES Only G or C base substitutions were observed (Sills et al., 1999). No A to T <i>ras</i> mutations observed in codon 61 (or codon 12 and 13)

² Chloroprene was tested in TA1535 in four different studies: 2 of which were positive, while 2 were negative. The positive studies used a different approach in the administration of chloroprene than the two negative studies. The question, unresolved at this time, is if the application method allowed for the degradation of chloroprene to reactive dimers, which have been shown to be mutagenic (Westphal et al. 1994).

- **Panel Members discussed the mutagenic activity of chloroprene but they did not seem to appreciate the lack of consistency in genotoxicity results. We believe that USEPA should reassess chloroprene to consider a non-genotoxic mode of action based on the following observations:**
 1. The results listed above challenge the weight of evidence presumption that chloroprene is genotoxic.
 2. Chloroprene is metabolized to an epoxide that binds with a high degree of specificity for G (guanine) and C (cytosine) DNA bases in a cell-free system (Munter et al., 2002, 2007). The USEPA interpretation that the excess A to T transversions found in lung tumor oncogenes is due to the mutagenic activity of the chloroprene epoxide conflicts with its established specificity for either G or C sites. Additionally, the absence of a dose-dependent increase in *ras* mutations concordant with the dose dependent increase in lung tumors (Sills et al., 1999) challenges the assumption that epoxide-induced *ras* mutations are the primary driver of lung tumors in the NTP mouse study.
 3. Scientists from the NTP and NIEHS have classified chloroprene as a non-genotoxic agent (Tennant et al. 2001; Prichard et al. 2003). Further, chloroprene did not produce tumors in Tg.AC and p53[±] transgenic mice strains (by the inhalation route) (Tennant et al. 2001; Prichard et al. 2003). The Tg.AC mouse screening test primarily responds to dermal applications of chemicals and does not necessarily distinguish genotoxic from non-genotoxic agents. In contrast, p53-null (heterozygous) mice respond well to genotoxic agents but not non-genotoxic agents (Prichard et al. 2002). The lack of a positive response in the p53-null mice is another piece of evidence that chloroprene is not acting by a genotoxic mode of action in the production of mouse tumors.
 4. Chloroprene produces hyperplasia in mouse bronchiolar and forestomach tissues. The incidence of hyperplasia increases with dose level, as does the incidence of alveolar/bronchiolar lung tumors.
 5. Results from the recently completed IISRP Genomics study (Himmelstein, personal communication) showed that chloroprene exposure results in dose related increases in cell proliferation in mouse lung bronchioles, but not in mouse lung alveoli.
 6. Induction of cell proliferation leading to hyperplasia with a secondary action of mutation expression is a scientifically recognized mode of action for toxic, but non-genotoxic carcinogens.

Toxicokinetics

- **Species differences in metabolism were previously published (Himmelstein, 2004a) but the Panel Members seemed to be unaware of key metabolism data.**

Himmelstein et al. (2004a) provided important information regarding species differences in metabolism. The reactions studied included total oxidative metabolism of chloroprene (which was used for PBTK modeling), simultaneous appearance of (1-chloroethenyl)oxirane, and detoxification reactions by microsomal epoxide hydrolase and cytosolic GST metabolism. Oxidation/hydrolysis ratios showed a 12-fold higher rate for mouse liver and 160-fold higher rate for mouse lung microsomes compared with human liver and lung microsomes, respectively (Himmelstein et al. 2004a). As noted in our written comments:

As a whole, the balance of reactive metabolite formation and detoxification across species appears to indicate that the mouse would be the most sensitive species, based on higher rates of epoxide formation, slower hydrolysis, and faster GSH conjugation, with perhaps the latter leading to an imbalance in glutathione (antioxidative) status and subsequently contributing to cytotoxicity.

In conclusion, these observations show that the mouse is not the most appropriate animal model for use in quantifying potential for cancer risk in the human.

- **One Panel Member made specific comments regarding the prevalence of polymorphisms in Glutathione S-Transferases (GST) in the US population and how these polymorphisms, specifically GST-null, may result in a sensitive subpopulation for cancer risk or disease.**

While it has been hypothesized that the presence of these null genotypes may increase the susceptibility of individuals to certain types of cancer or other diseases, several studies demonstrated no statistically significant association between the frequency of individual null genotypes and various cancers or diseases (Uzunoglu et al. 2006; Ho et al. 2006; Lizard-Nacol et al. 1999; Onaran et al. 2000; Bathi et al. 2009). Therefore, the presence of a GSTnull genotype does not always indicate an increased risk for disease.

Dose-Response Assessment – Noncancer

- **One of the Panel Members indicated that Chloroprene should be a Category 3 gas, not a Category 1, based on the USEPA (1994) RfC Dosimetry Guidelines.**

The toxicokinetics data that demonstrate systemic delivery of chloroprene provide support for Panel Member's position that chloroprene should be considered as a category 3 gas and not as a category 1 gas. This change in categorization impacts the dosimetric adjustment factors (DAFs) used to derive human equivalent concentrations (HECs) for the points of departure (POD_{HEC}) in Table 5-2 of the draft report. For the lung and systemic effects, we recommend that USEPA should use the PBTK model to estimate the POD_{HEC} values.

Dose-Response Assessment - Carcinogenicity

- **On the method of combining tumors, USEPA's practice of summing potency estimates for each tumor site assessed separately invokes an assumption of mutual exclusivity that is inappropriate and effectively results in double-counting of tumor-bearing animals.**

One of the authors of the Draft Toxicological Review provided the NAS (1994) report as justification for the approach used to sum unit risks. However, the NAS (1994) report also states that, "This procedure should be used unless specific data indicate that occurrence of the different tumor types within individual animals are significantly correlated." Application of the Kendall tau test for correlations to the individual tumor incidence data for chloroprene in both the male and female mouse suggests that significant correlations are present (correlation coefficients of ≥ 0.145 and p values ≤ 0.041); therefore USEPA's approach is not valid. We recommend that USEPA rely

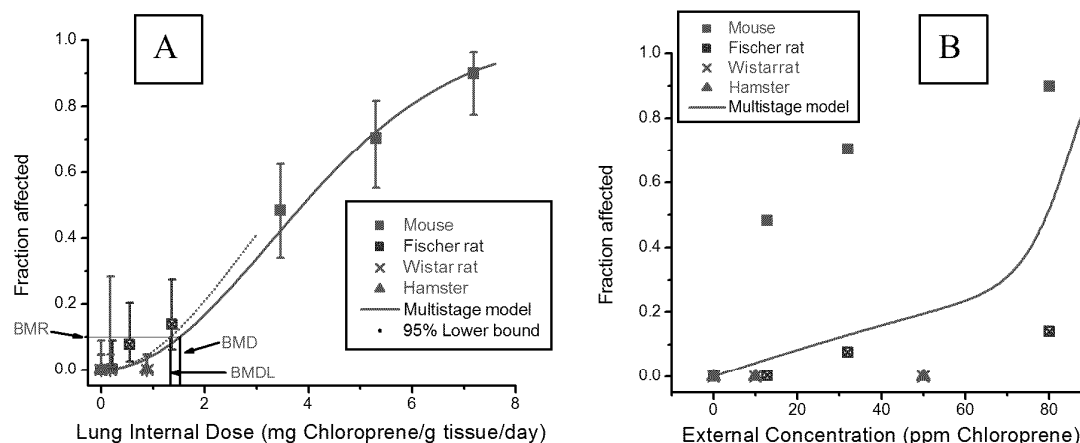
upon the most sensitive and relevant tumor type and that if any combining is to be performed, it should be done at the individual animal data level and not at the cancer potency level.

- **One of the Panel Members suggested the application of a mathematical approach that implied the “saturation” of tumor response could be modeled with a “Vmax and Km” approach that is empirical in nature and does not rely on PBTK modeling.**

The modeling approach presented at the Panel meeting appears to be based on the assumption that the overall shape of the tumor dose response curve was directly related to saturable metabolism of chloroprene. The approach discussed involved combining tumors and curve fitting using metabolic parameters (Vmax and Km) derived from the overall shape of the dose response curve. As mentioned in the DPE written comments, the combination of tumors as conducted by the Panel Member is not appropriate and can double count animals. Also, published kinetic data suggests that oxidative metabolism (as amount of metabolism per gram of liver or lung per day) is linear in the mouse up through the highest exposure concentration (80 ppm) used for the NTP inhalation bioassay (Himmelstein, 2004b). Therefore, use of a curve fitting approach that assumes metabolic saturation at a concentration of less than 80 ppm is inconsistent with the published literature on the metabolism of chloroprene.

- **One Panel Member discussed the continued use of external concentration in the derivation of the cancer potency estimate.**

As demonstrated in Himmelstein et al. (2004b), use of external concentration results in a cancer potency estimate that does not accurately predict observed tumor responses in exposed rats and hamsters, and therefore is not expected to reasonably predict tumor response in humans. These differences are readily visualized by comparison of the improved fit of the lung tumor dose response profiles for chloroprene using internal dose (panel A) versus external concentration (panel B) as shown below.



Use of a PBTK model-derived internal dose measure unifies responses such that all three animal species can be described on a single dose-response curve. The internal dose-response curve for lung tumors using all three species provides a scientifically sound basis for estimating potential risks to human populations exposed to chloroprene as explained in the written comments provided by DPE.

Comments made by Panel Members indicated confusion about why total metabolism of chloroprene would be a relevant dose metric for use in the dose-response assessment. Himmelstein et al. (2004a) measured total chloroprene oxidation (by disappearance kinetics). This captures the rate of metabolism to the known epoxide (1-chloroethenyl)oxirane and other unidentified (potentially reactive) metabolites. The “total” rate was scaled to the whole tissue, liver or lung, for incorporation into the *in vivo* PBTK model.

- **One Panel Member implied that the area under the blood concentration curve (AUC) of the identified reactive metabolite would be the more “correct” dose metric to use.**

The selection of the dose metric in Himmelstein et al. (2004b) was strongly influenced by early research showing that (1-chloroethenyl)oxirane is not detectable in the blood of mice or rats exposed to chloroprene by inhalation using the same highly sensitive gas chromatography-mass selective detection method used for the *in vitro* metabolism work (limit of quantitation ~0.06 μ M (1-chloroethenyl)oxirane in solution). Furthermore, Hurst and Ali (2007) while investigating hemoglobin adduct formation, showed that *in vitro* incubation of the *S*- and *R*-enantiomers of (1-chloroethenyl)oxirane with fresh mouse blood resulted in significant preferential GST-mediated enzymatic reactivity of the *S*-enantiomer with GSH. Thus, in addition to formation *in vivo* and detoxification in the tissue of origin (e.g., lung or liver), any epoxide presented to the circulation would be subject to detoxification in blood. This finding helps explain the lack of detection in blood and pragmatically precluded the development of data to support a metabolite based sub-model and AUC estimates as discussed by the Panel. Finally, Clewell et al. (2002), which recommended reactive metabolite formation (per volume of tissue per time) as the most appropriate dose metric when all the individual reactive metabolites are not known, leads to the conclusion that use of total metabolism is both technically achievable and a defensible foundation for the chloroprene dose metric for dose-response assessment.

- **Panel Members discussed the potential lack of relevance of the mouse lung tumors to human health.**

The issue of the relevance of the bronchioloalveolar tumors in the mouse to human health was raised by multiple Panel Members, given the documented differences in metabolism between the mouse, rat and human. There are differences in the incidence of morphologic subtypes of lung carcinomas in rodents versus humans (both spontaneous and chemically induced). Rat and mouse lung tumors typically exhibit local epithelial cell hyperplasia, involving Clara or alveolar type 2 cells lining the alveoli, with subsequent adenoma formation (Witschi 2005; Maronpot et al. 2004; Richards and Oreffo 1993). These appear as distinct tumors with uniformly solid or, occasionally, papillary adenomatous patterns and may progress to bronchioloalveolar adenocarcinomas; however, they infrequently metastasize. In contrast, human lung tumors are characteristically either undifferentiated small cell or non-small cell carcinomas; among the latter, adenocarcinoma (most frequently observed), squamous cell carcinoma, and large cell carcinomas can occur. Most of these tumors originate in the conducting airways (loosely characterized as bronchogenic carcinomas) and are typically highly invasive. Bronchioloalveolar carcinomas represent <10% of the total human lung cancer types. For these reasons, the relevance of the observed rat/mouse bronchioloalveolar tumors may be questioned for relevance to humans.

The current USEPA (2005) Guidelines for Carcinogen Assessment provide a framework for consideration of the relevance to human health of observations in animals. As a part of this framework, the similarity of metabolic activation and detoxification for a specific chemical between humans and tested species should be considered. Given the differences between humans and rodents for lung cancer, the USEPA should reconsider whether selection of mouse lung tumors as the most sensitive species/effect for unit risk calculations satisfies the framework guidance.

In conclusion, we consider our collective comments provide support for the conduct of a linear and nonlinear dose-response assessment. Should USEPA disagree with this position, we request that USEPA include these analyses nonetheless so readers can appreciate the difference in outcomes these alternative approaches would produce in the risk assessment. In either case, a PBTK model must be used to develop HECs. We hope that you will thoughtfully weigh these points, along with our written comments, in your consideration of the Peer Reviewer comments.

Sincerely yours,



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Cross-Species Transcriptomic Analysis of Mouse and Rat Lung Exposed to Chloroprene

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β-Chloroprene (2-chloro-1,3-butadiene), a monomer used in the production of neoprene elastomers, is of regulatory interest due to the production of multiorgan tumors in mouse and rat cancer bioassays. A significant increase in female mouse lung tumors was observed at the lowest exposure concentration of 12.8 ppm, whereas a small, but not statistically significant increase was observed in female rats only at the highest exposure concentration of 80 ppm. The metabolism of chloroprene results in the generation of reactive epoxides, and the rate of overall chloroprene metabolism is highly species dependent. To identify potential key events in the mode of action of chloroprene lung tumorigenesis, dose-response and time-course gene expression microarray measurements were made in the lungs of female mice and female rats. The gene expression changes were analyzed using both a traditional ANOVA approach followed by pathway enrichment analysis and a pathway-based benchmark dose (BMD) analysis approach. Pathways related to glutathione biosynthesis and metabolism were the primary pathways consistent with cross-species differences in tumor incidence. Transcriptional BMD values for the pathway were more similar to differences in tumor response than were estimated target tissue dose surrogates based on the total amount of chloroprene metabolized per unit mass of lung tissue per day. The closer correspondence of the transcriptional changes with the tumor response is likely due to their reflection of the overall balance between metabolic activation and detoxication reactions, whereas the current tissue dose surrogate reflects only oxidative metabolism.

Key Words: transcriptomics; microarrays; 2-chloro-1,3-butadiene; chloroprene; lung; mice; rats.

Chloroprene (β-chloroprene, 2-chloro-1,3-butadiene) is a volatile, chlorinated hydrocarbon monomer used in the manufacture of polychloroprene synthetic rubber (Lynch, M, 2001). Occupational exposure to chloroprene occurs primarily through inhalation and can occur during monomer synthesis, transport, and polymerization (Lynch, J, 2001). Because of its structural

similarity to butadiene and vinyl chloride, studies on the health effects of chloroprene in humans have focused on the potential carcinogenicity of chloroprene in the liver, lung, and lymphohematopoietic systems (Bukowski, 2009). A recent review of epidemiological studies does not support a link between chloroprene exposure and human cancer; however, inconsistencies in the studies and the lack of controls for major confounders limit the ability to draw firm conclusions on causality (Bukowski, 2009).

Multiple acute, subchronic, and chronic animal studies have been performed to gain understanding of possible adverse health effects of chloroprene in humans (EPA, 2010a, b; Pagan, 2007). For cancer-related effects, 2-year cancer bioassays have been performed in mice, two strains of rats, and hamsters (NTP, 1998; Trochimowicz *et al.*, 1998). The bioassays showed significant strain and species differences in sensitivity and organ specificity. In B6C3F1 mice, treatment-related tumors were observed in the lung, circulatory system, Harderian gland, forestomach, kidney, mammary gland, skin, mesentery, Zymbal gland, and liver. In rats, treatment-related tumors were observed in the lung, oral cavity, thyroid gland, kidney, and mammary gland of the Fischer 344 (F344) strain, whereas the Wistar strain only showed a weak response in mammary tissue and no other organs. No treatment-related tumors were observed in Syrian hamsters. Specifically for the lung, both male and female B6C3F1 mice showed increased incidences of alveolar/bronchiolar adenomas and alveolar/bronchiolar carcinomas in all exposed groups. In female F344 rats, the incidences of alveolar/bronchiolar carcinomas and alveolar/bronchiolar adenomas or carcinomas (combined) were slightly elevated at 80 ppm, but not statistically significant.

The current hypothesized mode of action for chloroprene involves bioactivation to a mutagenic metabolite, leading to DNA damage and increased tumors (EPA, 2010b; Pagan 2007). Although the specific carcinogenic metabolite(s) are currently unknown, chloroprene is oxidized by cytochrome

P450 enzymes to several metabolites, some of which react with DNA and induce mutations (Munter *et al.*, 2007). The primary isozyme responsible for chloroprene oxidation is Cyp2e1 (Cottrell *et al.*, 2001; Himmelstein *et al.*, 2001a). The metabolite profile is qualitatively similar across species, but quantitative differences exist in the rates of oxidative metabolism and in the downstream detoxification steps (Himmelstein *et al.*, 2004b; Munter *et al.*, 2007). The rate of oxidative metabolism in liver microsomes was slightly faster in the mouse and hamster than in the rat or human, whereas in lung microsomes, the rate of oxidative metabolism was much greater in mice than other species (Himmelstein *et al.*, 2004b). Two oxidative metabolites of interest are the epoxides, (1-chloroethenyl)oxirane and 2-chloro-2-ethenyloxirane (Cottrell *et al.*, 2001; Himmelstein *et al.*, 2001a). The 2-chloro-2-ethenyloxirane metabolite was very unstable and rapidly converted to ketone and aldehyde products. The (1-chloroethenyl)oxirane was mutagenic in *Salmonella typhimurium* but not clastogenic at noncytotoxic concentrations (Himmelstein *et al.*, 2001b). The (1-chloroethenyl)oxirane epoxide also shows reactivity with DNA *in vitro* and is a potential cross-linking agent (Munter *et al.*, 2002; Wadugu *et al.*, 2010). Detoxification of (1-chloroethenyl)oxirane through epoxide hydrolase was faster in human and hamster microsomes than in rat or mouse (Himmelstein *et al.*, 2004b). Overall, physiologically based pharmacokinetic (PBPK) modeling has suggested that the species differences in the total amount of chloroprene metabolized per gram of lung tissue per day can, at least in part, account for the difference in lung tumor incidence across species (Himmelstein *et al.*, 2004a).

Comparative biology is an important tool for understanding organism diversity and inferring conservation of phenotypic responses across species. In toxicology, two fundamental, but interrelated, challenges are to identify a mode of action for a particular chemical and determine whether a particular response will be conserved across species. The determination of whether a response will be conserved usually involves a mechanistic understanding of the molecular events and an assessment of whether the processes and components that comprise those events are present in both the model species and the species of interest. In a previous study, dose-response changes in gene expression associated with specific pathways were highly correlated with both noncancer and cancer pathological responses, and many of the correlated pathways were relevant to disease pathogenesis (Thomas *et al.*, 2012). In a separate study, gene expression changes were used to assess cross-species differences in potency and efficacy in response to dioxin treatment (Black *et al.*, 2012). The results showed divergent cross-species gene expression changes in response to treatment, which were consistent with epidemiological and clinical evidence showing humans to be less sensitive to the compounds examined. Taken together, the previous studies suggest that cross-species transcriptomic studies using a combination of sensitive and insensitive species may be used to identify potential modes of action for a chemical.

In this study, gene expression microarray measurements were made in the lungs of female B6C3F1 mice and female F344 rats, respectively, as the sensitive and insensitive species to chloroprene-induced lung tumors. Focus was given to the lung because it is the site of chloroprene uptake and the most sensitive tissue for tumor development, particularly in the mouse. The exposure concentrations were selected to span those used for the cancer bioassay in the mouse and extend the concentration range in the rat. The concentration range was extended in the rat to provide approximately matched tissue dose surrogates based on the total amount of chloroprene metabolized per gram of lung tissue per day. Exposures were performed for 6 h/day for 5 or 15 days. The gene expression changes were analyzed using both a traditional ANOVA approach followed by pathway enrichment analysis and a pathway-based benchmark dose (BMD) analysis approach (Thomas *et al.*, 2012).

MATERIALS AND METHODS

Chemical and purification. Chloroprene (CAS 126-99-8), containing *p*-tertiary butyl catechol as a stabilizing agent, was supplied by E.I. DuPont de Nemours and Company, Inc. as a clear liquid (99% purity). Removal of the *p*-tertiary catechol was accomplished by passing approximately 500 ml of stabilized chloroprene through a 100-ml activated alumina column in a nitrogen atmosphere (ICN Biomedicals, Eschwege, Germany) (Himmelstein *et al.*, 2001a). The first 10–20 ml of chloroprene to elute from the alumina column was discarded. The remaining purified chloroprene was collected in multiple screw cap glass vials of either 20 or 40 ml volume sizes. Vials were filled to approximately 90% of capacity, tightly capped, taped shut using electrical tape, and labeled. Capped and sealed vials were placed in metal cans to protect from light (5–6 vials per can) and stored at -70°C until required. Analytical chemistry confirmed that the impurities were $< 1\%$.

Animals and exposures. Female B6C3F1/Crl mice and female F344/NCrI rats were acquired from Charles River at approximately 8 weeks of age. The animals were acclimated approximately 10 days prior to exposure to test chemical and were approximately 10 weeks old at the start of the exposure. Animals were housed in a temperature (18.3°C – 23.9°C) and humidity (40–60%)–controlled environment with a standard 12-h light/dark cycle (approximately 0700–1900 h for the light phase). Air flow within the housing environment was maintained at 12 to 15 air changes per hour. NIH-07 diet (Zeigler Bros., Gardners, PA) was provided *ad libitum*, except during inhalation exposures. Reverse osmosis purified water was available *ad libitum* to all animals.

Approximately 1 week after arrival, the animals were weighed, randomized, ear tagged, and randomly assigned to treatment groups. The treatment groups for the mice included groups receiving 0.3, 3, 13, and 90 ppm chloroprene and a sham air control (0 ppm). The treatment groups for the rats included groups receiving 5, 30, 90, and 200 ppm chloroprene and a sham air control (0 ppm). The exposure concentrations were selected to span those used for the cancer bioassay in the mouse and extend the concentration range in the rat. The animals were exposed 6 h/day, 5 day/week in 1-m³ whole-body inhalation chambers (Hazelton H1000, Lab Products, Seaford, DE) constructed of glass and stainless steel. A separate 1-m³ chamber was used for each exposure concentration and the control. Both the mice and rats were treated for 5 days (5 days of exposure) and 19 days (15 exposure days).

Animal use in this study was approved by the Institutional Animal Use and Care Committee of The Hamner Institutes for Health Sciences and was conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Animals were housed in fully accredited American Association for Accreditation of Laboratory Animal Care facilities.

Atmosphere generation and monitoring. Exposure atmospheres were generated by metering saturated chloroprene vapor from a stainless steel pressure vessel reservoir into the exposure chamber air supply. A small volume of chloroprene was injected in the bottom of a pressure vessel, which was then pressurized with ultrahigh purity nitrogen (National Welders Supply Co., Inc., Durham, NC). After an overnight equilibration period, the concentrated chloroprene vapor was metered through a mass flow controller (MKS Instruments Inc., Andover, MA) into the chamber air inlet. Although not specifically analyzed, chloroprene dimer formation was not expected using this vapor generation method. The concentration in the exposure system was adjusted by changing the flow through the mass flow controller.

Exposure atmospheres were analyzed by a Hewlett Packard 5890 gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) equipped with a flame ionization detector and 10-port sampling valve (VICI Valco Instruments, Houston, TX). Exposure atmospheres were continuously pulled from each chamber. A 10-port sampling valve rotated through a series of ports connected to the chamber sample lines to inject a sample from each port onto the GC. The GC analysis was timed so that the valve rotated through all 10 positions in 60 min during the exposure period. GC chromatograms of samples taken from the inhalation exposure chambers were inspected visually for appearance of additional peaks as a quality check for test substance stability throughout the study period. Other than the peak for chloroprene, no additional peaks appeared following visual inspection of the GC chromatograms during daily exposures, indicating stability of the test substance throughout the study. The calculated limit of detection was 0.18 and 0.27 ppm for the mouse and rat exposures, respectively. The exposure chamber distribution uniformity measurements were performed after the end of the study for an exposure concentration of 200 ppm. The coefficient of variation was less than 3% for the exposure chamber used for the 200-ppm exposure group.

Necropsy and histology. Animals were sacrificed immediately following exposure on the 5th day (5 days of exposure) or the 19th day (15 exposure days). Animals were exposed in the morning and sacrificed in the afternoon. To allow sufficient time for off-gassing in the treated animals, the control animals were sacrificed first. Among the treated animals, the animals were sacrificed in a staged order with one animal sacrificed sequentially from each concentration (i.e., one animal from 0.3 ppm, one animal from 3 ppm, etc. until the highest concentration, and then the process was repeated). The animals were anesthetized with sodium pentobarbital and exsanguinated. Gross observations were recorded in Provantis NT2000.

Lung samples were obtained by placing a suture loosely around the left bronchus at the site where the bronchus enters the left lung lobe. A second suture was placed around the right bronchus and pulled tightly to tie off the right lung lobes. The right lung lobes were removed and placed in RNAlater (Ambion, Austin, TX), and the left lung infused under hydrostatic pressure (~30 cm) with neutral buffered formalin. After the left lung was filled with fixative, the suture around the left bronchus was pulled tightly to tie off the expanded left lobe. The left lung lobe with trachea attached was removed from the carcass and transferred to a cup of neutral buffered formalin. Samples placed in RNAlater were incubated overnight at 4°C and frozen the next day at < -70°C. Following a standard fixation period, the formalin-fixed tissues were placed in cassettes for cross-sectional embedment into paraffin. The lungs were sectioned, stained with hematoxylin and eosin, and evaluated for histopathology. Histological changes were assessed by an accredited pathologist.

Gene expression microarray measurements. Gene expression microarray analysis was performed on five mice and five rats per concentration per time point. Total RNA was isolated from the lung tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). The isolated RNA was further purified using RNeasy columns (Qiagen, Valencia, CA) and the integrity of the RNA verified spectrophotometrically and with the Agilent 2100 Bioanalyzer (Palo Alto, CA). Double-stranded cDNA was synthesized from 5 µg of total RNA using the One-Cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was transcribed from the cDNA using the GeneChip IVT Labeling Kit (Affymetrix). Fifteen micrograms of labeled cRNA was fragmented and hybridized to Affymetrix Mouse Genome 430 2.0 or Rat Genome 230 2.0 microarrays. The hybridized arrays were washed using the GeneChip

Fluidics Station 450 and scanned using a GeneChip 3000 scanner. The gene expression results were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (Accession No.: GSE40795).

Gene expression ANOVA and pathway enrichment analysis. Microarray data were preprocessed using the robust multiarray average method with a log base 2 (\log_2) transformation (Irizarry *et al.*, 2003). The basic statistical analysis of the gene expression changes in each species were performed using ANOVA with contrasts between each chemical concentration and the associated control group at each time point (e.g., 0.3 ppm vs. control at 5-day time point). Probability values were adjusted for multiple comparisons using a false discovery rate (FDR) and significance was defined as $FDR < 0.05$ (Benjamini and Hochberg, 1995). The ANOVA analysis and associated contrasts were performed using Partek Genomics Suite (Partek Incorporated, St Louis, MO). The probe sets were converted into genes based on the annotation provided by Affymetrix. The pathway enrichment analysis was conducted using the GeneGo pathway maps in the Metacore database (Version 6.10 build 31731; GeneGo, St Joseph, MI). The GeneGo pathway maps included the ToxHunter pathways. The enrichment p values were calculated based on a hypergeometric distribution with the GeneGo database used as the background. Significant enrichment was defined as a FDR-corrected p value < 0.05 .

Transcriptional BMD analysis. The analysis of the gene expression microarray data was performed as described previously (Thomas *et al.*, 2007) with modification. All probe sets were fit as continuous data to a series of four different dose-response models—linear, 2° polynomial, 3° polynomial, and power models. Each model was run assuming constant variance, and the benchmark response (BMR) factor was set to 1.349 multiplied by the SD in the control animals to estimate a BMD with a 10% increase in tail area (see Thomas *et al.* [2007] for details on its derivation). For model selection, a nested likelihood ratio test was performed on the linear, 2° polynomial, and 3° polynomial models. If the more complex model provided a significantly improved fit ($p < 0.05$), the more complex model was selected. If the more complex model did not provide a significantly improved fit ($p \geq 0.05$), the simpler model was selected (Posada and Buckley, 2004). The Akaike information criterion (AIC) for the selected polynomial model was then compared with the AIC for the power model. The model with the lowest AIC (Akaike, 1973) was selected as the final model and was used to calculate a BMD and the associated lower 95% confidence level (BMDL). To avoid model extrapolation and any potential bias from poor fitting genes, probe sets with a BMD value greater than the highest concentration or a goodness-of-fit p value < 0.1 were removed from further analysis.

Affymetrix probe sets were converted into unique genes based on their NCBI Entrez Gene ID. Promiscuous probe sets (i.e., those that interrogate more than one gene) were removed from the analysis. When two or more probe sets were associated with a single gene, the BMD and BMDL values for the individual probe sets were averaged to obtain a single BMD and BMDL value. The Entrez Gene identifiers were then matched to their corresponding pathways using the GeneGo Metacore database (GeneGo, St Joseph, MI). A total of 682 pathways were used, which included the GeneGo pathway maps with the ToxHunter supplemental pathways. Pathways with less than five genes were removed from the analysis. Unless otherwise stated, median BMD and BMDL values were used to summarize each pathway.

BMD analysis of lung tumor incidence. The data set containing the combined incidences of adenoma and carcinoma in the lung (4/50, 28/49, 34/50, 42/50) from the National Toxicology Program 2-year rodent cancer bioassay was fit to the multistage cancer model with a BMR of 10% extra risk (BMDS software, Version 2.1.2, U.S. Environmental Protection Agency). For the mouse lung tumor data set, a linear model was used to fit these responses; however, the fit of the model was poor ($p = 0.044$). Dropping the high dose marginally improved the fit ($p = 0.047$) but had little effect on the BMD or BMDL. This analysis was different from the time-to-tumor analysis employed by the U.S. Environmental Protection Agency (U.S. EPA) in the Integrated Risk Information System (IRIS) risk assessment.

For the rat lung tumor data set (1/49, 0/50, 0/50, 3/50), a third-order polynomial was selected as the best model based on the AIC. The goodness-of-fit p

value indicated a good fit ($p = 0.333$), but the BMD estimate was greater than the highest dose used in the bioassay. The U.S. EPA did not perform an analysis of the rat lung tumor data in their IRIS assessment.

Pathway filtering based on cross-species differences in lung tumor responses. To identify potential key events in the mode of action of chloroprene lung tumorigenesis, both the pathway enrichment and transcriptional BMD results were filtered to identify those whose dose-response changes were consistent with the cross-species differences in tumor incidence. The pathway enrichment results were filtered based on the following criteria: (Mouse Pathway Enrichment at 13 ppm at 5-day time point OR Mouse Pathway Enrichment at 13 ppm at 15-day time point) AND (NOT Rat Pathway Enrichment < 90 ppm at 5-day time point) AND (NOT Rat Pathway Enrichment < 90 ppm at 15-day time point). The transcriptional BMD results were filtered based on the following criteria: (Mouse Pathway BMD ≤ 10 ppm at 5-day time point OR Mouse Pathway Enrichment ≤ 10 ppm at 15-day time point) AND (NOT Rat Pathway BMD < 80 ppm at 5-day time point) AND (NOT Rat Pathway BMD < 80 ppm at 15-day time point).

Target tissue dose surrogate calculation. A modified PBPK model for chloroprene in the rat and mouse (Himmelstein *et al.*, 2004a; Yang *et al.*, 2012) was used to calculate target tissue (lung) dose surrogates for the putative carcinogenic moieties, the reactive epoxides produced by the metabolism of chloroprene in the lung. As in previous studies (Andersen *et al.*, 1987; Clewell *et al.*, 2001), the surrogate used for the target tissue exposure to reactive metabolites was the predicted total amount of parent chemical (chloroprene) metabolized by the target tissue per gram of tissue per day. The lung and liver metabolic parameters in the PBPK model were estimated using *in vitro* data on the metabolism of chloroprene in pooled microsomes (Himmelstein *et al.*, 2004a; Yang *et al.*, 2012). Significantly higher metabolic capacity per gram lung was estimated in the female mouse than in the female rat (0.11 and 0.014 mg/h/kg, respectively). The Michaelis constant (K_m) in the mouse lung was estimated to be 0.25 mg/l; K_m in the rat lung was not identifiable, so the estimated rat liver K_m value of 0.09 was used for the rat lung as well. To obtain the dose surrogates, the model was run to simulate the exposure pattern in the experimental study for the period of interest (5 or 15 days), and the predicted total metabolism per gram of lung tissue was divided by the number of days simulated.

RESULTS

Histological Changes

At necropsy, there were no gross observations for animals in this study. Minimal epithelial hyperplasia of the terminal bronchioles was evident in the majority of the mice exposed to 90 ppm chloroprene following 5 or 15 days duration (seven out of eight mice at 5 days and eight out of eight mice at 15 days). This was a subtle effect and characterized by an increased number of respiratory epithelial cells with occasional large nuclei in the terminal bronchioles. There were no treatment-related microscopic effects observed in the lungs of rats exposed to up to 200 ppm chloroprene for up to 15 days compared with control rats.

Gene Expression Changes Based on ANOVA

In the female mouse lung, the number of genes showing significant change in expression (either up- or downregulation) (± 1.5 -fold and $FDR < 0.05$) increased with concentration at both the 5- and 15-day time points (Table 1). No genes showed significant changes at the 0.3 ppm concentration at either time point. At the 5-day time point, a total of 28, 60, and 725 genes showed significant changes in expression at 3, 13, and 90 ppm concentrations, respectively. At the 15-day time point, a total of 15, 53, and 716 genes showed differential expression at the

TABLE 1
Total Number of Differentially Expressed Probes and Genes
(+ 1.5-fold change and $FDR < 0.05$)

Mouse			Rat		
Concentration (ppm)	Time point		Concentration (ppm)	Time point	
	5 days	15 days		5 days	15 days
0.3	0 (0) ^a	0 (0)	5	0 (0)	0 (0)
3	31 (28)	20 (15)	30	0 (0)	178 (72)
13	79 (60)	76 (53)	90	563 (259)	119 (62)
90	984 (725)	987 (716)	200	800 (395)	195 (108)

^aNumber of probes (genes).

same exposure concentrations. Responses were approximately equally divided between upregulated and downregulated genes. Hierarchical clustering of all significant genes showed that most genes possessed similar dose-response changes at the 5- and 15-day time points (Fig. 1A). The full set of differentially expressed mouse genes and probes is available as supplementary material (Supplementary File 1).

In the female rat lung, no significant changes in the expression of any genes were observed either at the 5 ppm concentration at the 5- or 15-day time point or at the 30 ppm concentration at the 5-day time point. The number of genes with significantly altered expression in the rat lung at the 5-day time point was 259 and 395 at the 90 and 200 ppm concentrations, respectively (Table 1). At the 15-day time point, the number of genes with significantly altered expression was 72, 62, and 108 at 30, 90, and 200 ppm, respectively. In contrast to the mouse, the majority of the genes showing differential expression were upregulated and the response tended to be more acute (i.e., larger number of differentially expressed genes at the 5-day time point compared with the 15-day time point). Hierarchical clustering of the differentially expressed genes showed that a large proportion displayed differential behavior at the two time points (Fig. 1B). The full set of differentially expressed rat genes and probes is available as supplementary material (Supplementary File 1).

Pathway Enrichment Analysis

Among the top 10 pathways significantly enriched in the female mouse lung, the Nuclear factor (erythroid-derived 2)-like 2 (NRF2) pathway related to oxidative stress and the glutathione metabolism pathway were enriched beginning at 3 ppm and extending to the highest concentration (90 ppm) at both time points (Fig. 2). The extent of enrichment was not dose dependent for these pathways because the 13 ppm dose showed the largest $-\log p$ value at both time points. The genes associated with enrichment of the glutathione pathway included those involved in biosynthesis and metabolism (Supplementary File 2). Significant enrichment was also observed in the pathway related to the effect of arsenite on glucose-stimulated insulin secretion beginning at 3 ppm and both time points except the 90 ppm concentration at 5 days. Significant enrichment in immune

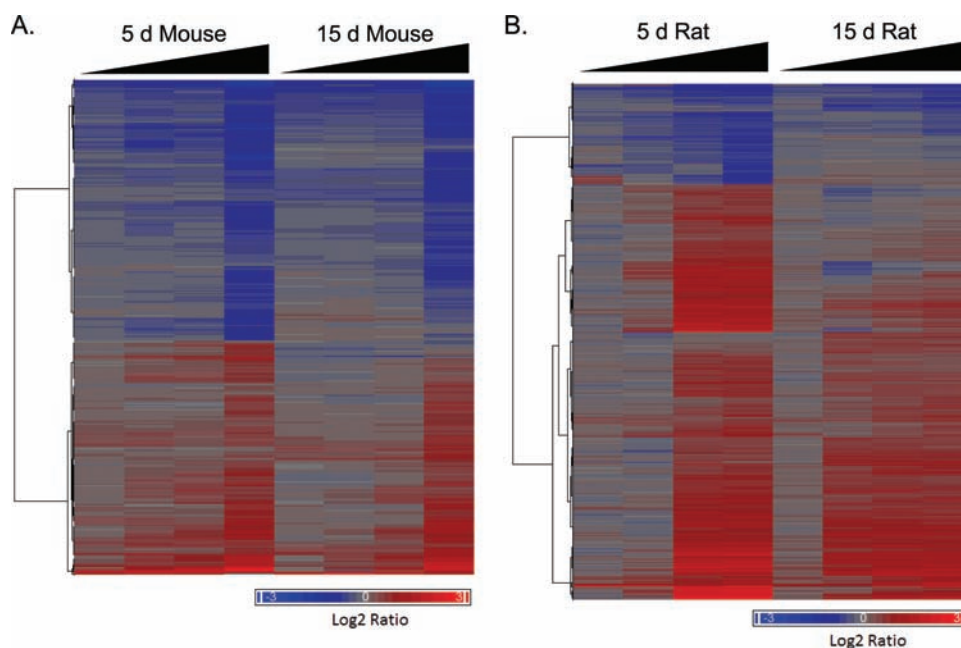


FIG. 1. Hierarchical clustering of differentially expressed probe sets (fold-change $> \pm 1.5$ and $\text{FDR} < 0.05$) in the (A) mouse and (B) rat lung as a function of dose and time. The color and intensity represent the \log_2 fold change in expression relative to the vehicle control at the associated time point. The columns in the heat map are arranged from left to right with increasing concentrations of chloroprene (0.3, 3, 13, and 90 ppm in the mouse and 5, 30, 90, and 200 ppm in the rat). Clustering was performed using Euclidean distance and average linkage.

response pathways occurred only at the highest concentration at both time points. The immune response pathways included the T-cell receptor pathway, PIP3 signaling in B lymphocytes, the B-cell receptor pathway, and T-cell receptor and CD28 stimulation of nuclear factor kappa B (NF κ B). The full set of enriched mouse pathways is available as supplementary material ([Supplementary File 3](#)).

Among the top 10 pathways significantly enriched in the female rat lung, both the circadian rhythm and cytoskeleton remodeling pathways were enriched beginning at 90 ppm at the 5-day time point and beginning at 30 ppm at the 15-day time point ([Fig. 3](#)). Significant enrichment was observed in the NRF2 pathway related to oxidative stress at the highest two concentrations at the 5-day time point and the highest concentration at the 15-day time point. Similarly, enrichment in the bone morphogenetic protein (BMP) signaling pathway was observed at the highest two concentrations at the 5-day time point, whereas enrichment in methionine, cysteine, and glutamate metabolism was observed at the highest two concentrations at the 15-day time point. The remaining pathways related to integrin signaling, the Jun N-terminal kinase (JNK) pathway, and epidermal growth factor (EGF) receptor signaling were only enriched at the 90 ppm concentration at the 5-day time point. The full set of enriched rat pathways is available as supplementary material ([Supplementary File 3](#)).

BMD Analysis of Lung Tumor Incidence

The data sets containing the combined incidences of adenoma and carcinoma in the lung from the National Toxicology Program 2-year rodent cancer bioassay were fit to the multi-stage cancer model with a BMR of 10% extra risk. The BMD_{10}

and BMDL_{10} for the female mouse lung tumors was 3.6 and 2.9 ppm, respectively. The time-to-tumor analysis employed by the U.S. EPA in the IRIS assessment resulted in a lower BMD_{10} and BMDL_{10} of 1.2 and 0.88 ppm, respectively. The BMD_{10} and BMDL_{10} for the female rat lung tumors were 102 and 76 ppm, respectively. The U.S. EPA did not perform an analysis on the rat tumor data. Based on our dose-response modeling, the cross-species ratio in BMD_{10} values was 28 (102 vs. 3.6 ppm).

Transcriptional BMD Analysis

In the female mouse lung, the most sensitive pathway at the 5-day time point related to nitric oxide signaling in survival had a median BMD of 1.83 ppm ([Table 2](#)). The next series of sensitive pathways at the 5-day time point had median BMD values that clustered between 5 and 7 ppm. These included pathways related to circulation of bile acids, glutathione metabolism, amino acid metabolism, and activation of the constitutive androstane receptor (CAR). At the 15-day time point, the most sensitive pathways had BMD values that clustered between 5 and 8 ppm. These included pathways related to glutathione metabolism, heme metabolism, circulation of bile acids, and ubiquinone metabolism. The full set of mouse transcriptional BMD results is included as supplementary material ([Supplementary File 4](#)).

In the rat lung, the most sensitive pathway at the 5-day time point was ascorbate metabolism with a median BMD of 37.99 ppm ([Table 3](#)). Other sensitive pathways at the 5-day time point had median BMD values between 40 and 50 ppm were related to glycosphingolipids metabolism, beta-alanine metabolism, neurite outgrowth, and DNA replication. At the 15-day time point, the BMD values for the most sensitive pathways were

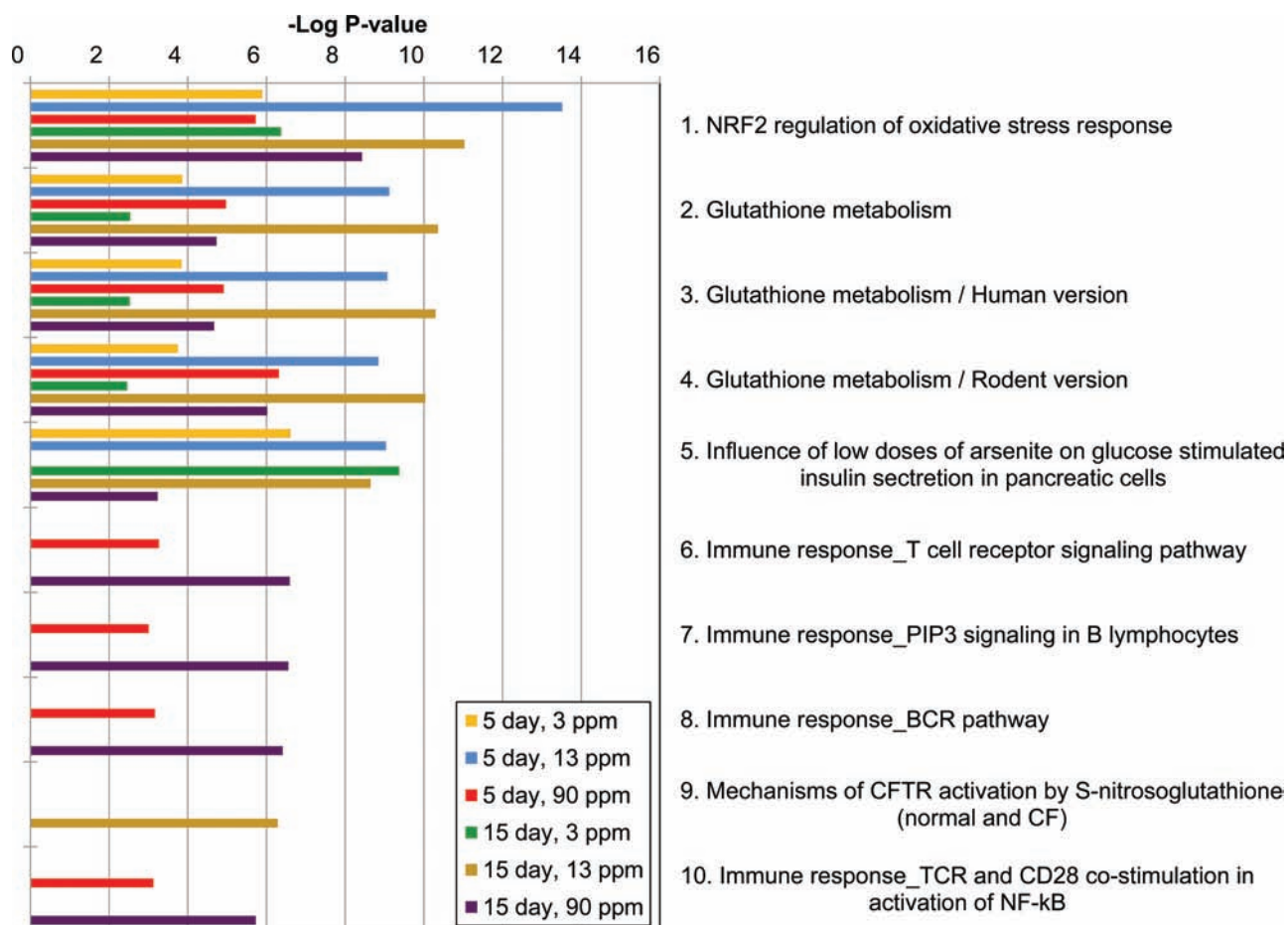


FIG. 2. Top 10 significantly enriched pathways in the mouse lung following exposure to chloroprene. Bars are color coded based on time point and chloroprene concentration. Missing bars indicate that the pathway was not significantly enriched (FDR < 0.05).

lower, ranging from 12 to 17 ppm. These included pathways related to growth hormone signaling, immune signaling, vitamin D receptor signaling, and thyroid hormone-related signaling. The full set of rat transcriptional BMD results is included as supplementary material (Supplementary File 4).

Pathway Responses Consistent With Cross-Species Tumor Incidence

To identify potential key events in the mode of action of chloroprene lung tumorigenesis, both the pathway and process enrichment analysis and the pathway BMD analysis were filtered to identify those whose dose-response changes were consistent with the cross-species differences in tumor incidence. In the enrichment analysis, a pathway must be enriched in mice at the lowest concentration in the 2-year cancer bioassay where a significant increase in lung tumors was observed (i.e., 12.8 ppm) and not enriched in female rats at the highest concentration tested in the 2-year cancer bioassay where no significant increase in lung tumors was observed (i.e., 80 ppm). Due to dose spacing in the study, this amounted to pathways being enriched at the 13 ppm concentration in the mouse lung at either the 5- or 15-day

time points. The pathways must also not be enriched in the rat at the 30 ppm concentration at either time point. Nothing was enriched at the 5 ppm concentration because no genes were altered. A total of 17 pathways met these criteria (Table 4).

A previous study demonstrated good concordance between pathway-based transcriptional BMD values and those for apical cancer and noncancer responses (Thomas *et al.*, 2011). In the chloroprene cancer bioassay, the BMD for female mouse lung tumors was 3.62 ppm, whereas the BMD for female rat lung tumors was greater than the highest dose used in the bioassay (80 ppm). To match these values within a reasonable range of experimental variation, the pathway-based transcriptional BMD values were filtered to include those with BMD values ≤ 10 ppm at either the 5- or 15-day time point in the mouse and BMD values > 80 ppm at both time points in the rat. A total of four pathways met these criteria (Table 5).

Dose Surrogates in the Mouse and Rat Lung Based on Pharmacokinetics

Because the specific carcinogenic metabolite(s) are currently unknown, a previous study used the total amount of oxidative

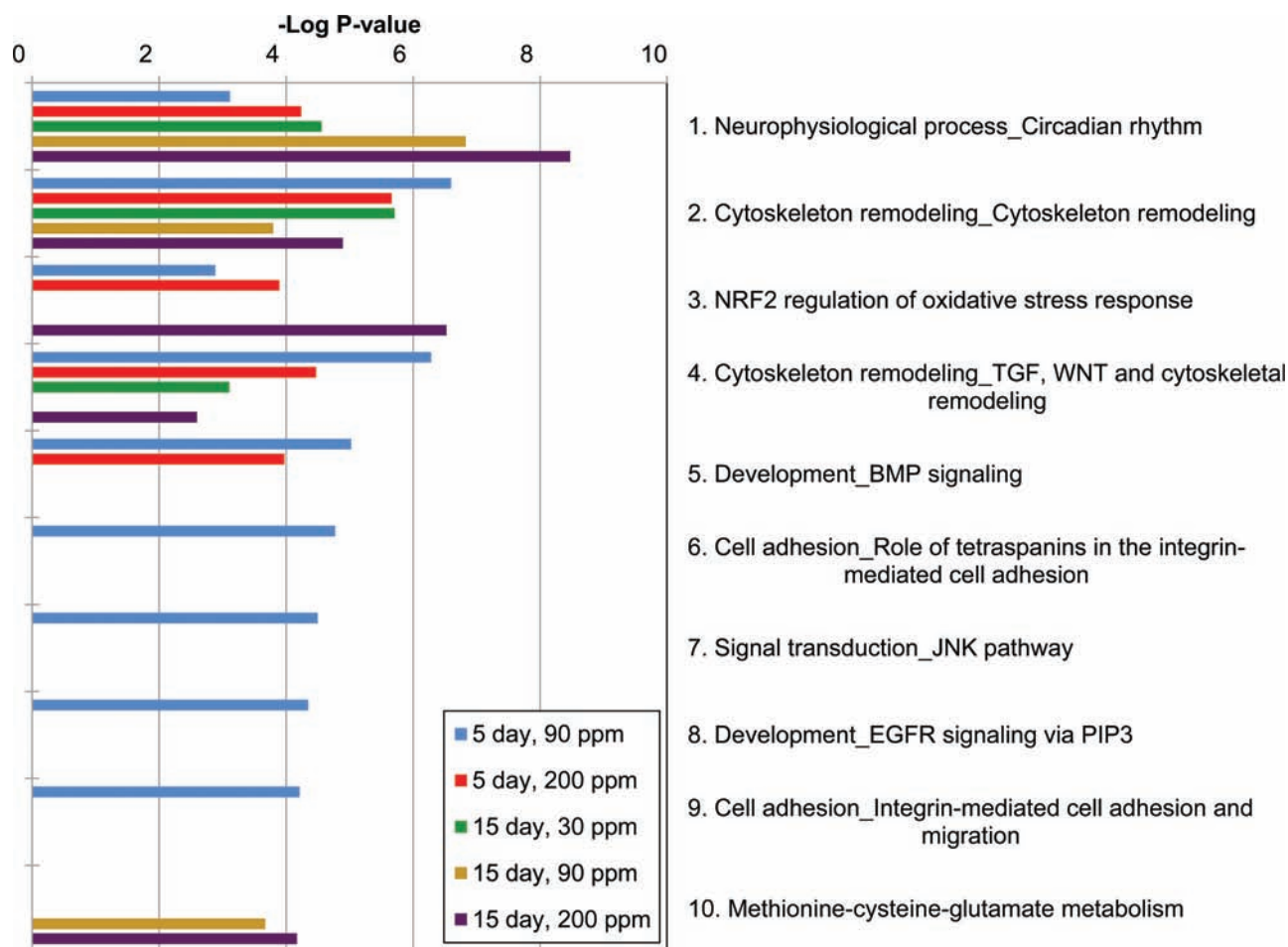


FIG. 3. Top 10 significantly enriched pathways in the rat lung following exposure to chloroprene. Bars are color coded based on time point and chloroprene concentration. Missing bars indicate that the pathway was not significantly enriched (FDR < 0.05).

TABLE 2
Transcriptional BMD Estimates for the Most Sensitive Pathways in the Mouse Lung

Pathway	Total genes in pathway/ genes with BMD	Median BMD (ppm)	Median BMDL (ppm)
5-day time point			
Apoptosis and survival: NO signaling in survival	20/7	1.83	1.12
Cholehepatic circulation of bile acids	16/5	5.68	4.00
Glutathione metabolism	36/21	5.85	3.80
Methionine-cysteine-glutamate metabolism	18/10	6.10	3.94
Constitutive androstane receptor-mediated direct regulation of xenobiotic metabolizing enzymes	27/8	7.54	4.90
15-day time point			
Glutathione metabolism	36/18	5.85	3.86
Heme metabolism	26/11	6.35	4.08
Enterohepatic circulation of bile acids	20/7	6.83	4.61
Ubiquinone metabolism	41/7	8.03	5.16
Tyrosine metabolism p.1 (dopamine)	16/5	11.10	6.43

metabolites per unit lung mass per day as the dose surrogate for scaling the potential lung cancer risk across species (Himmelstein *et al.*, 2004a). The previous model was refined based on updated *in*

vitro data (Yang *et al.*, 2012), and the total amount of chloroprene metabolized per unit lung mass was recalculated (Fig. 4). The results show a nonlinear relationship in the dose surrogate

TABLE 3
Transcriptional BMD Estimates for the Most Sensitive Pathways in the Rat Lung

Pathway	Total genes in pathway/ genes with BMD	Median BMD (ppm)	Median BMDL (ppm)
5-day time point			
Ascorbate metabolism	12/5	37.99	24.14
Globo-(isoglobo) series glycosphingolipids metabolism	11/5	42.95	25.93
Beta-alanine metabolism	10/5	45.83	27.06
Development: MAG-dependent inhibition of neurite outgrowth	50/13	49.00	28.24
Cell cycle: Start of DNA replication in early S phase	32/13	49.05	29.00
15-day time point			
Development: Growth hormone signaling via PI3K/AKT and MAPK cascades	41/7	12.84	8.04
Immune response: Lectin-induced complement pathway	24/5	15.02	8.93
Immune response: Oncostatin M signaling via MAPK	39/9	15.59	9.10
Transcription: Role of vitamin D receptor (VDR) in regulation of genes involved in osteoporosis	41/8	17.17	9.63
Regulation of metabolism: Triiodothyronine and thyroxine signaling	27/5	17.32	9.75

TABLE 4
Significantly Enriched Pathways Consistent With Cross-Species Differences in Lung Tumor Incidence

Pathway ^a	5-day time point	15-day time point
	Mouse–Log (<i>p</i> value)	Mouse–Log (<i>p</i> value)
NRF2 regulation of oxidative stress response	13.52	11.03
Glutathione metabolism	9.12	10.36
Influence of low doses of arsenite on glucose-stimulated insulin secretion in pancreatic cells	9.04	8.65
Transcription: Role of AP-1 in regulation of cellular metabolism	3.68	3.49
Methionine-cysteine-glutamate metabolism	3.51	4.82
Transcription: Transcription regulation of amino acid metabolism	2.57	2.45
Transcription: Role of Akt in hypoxia-induced hypoxia inducible factor 1 alpha (HIF1) activation	2.51	2.39
Constitutive androstane receptor-mediated direct regulation of xenobiotic metabolizing enzymes	2.15	2.03
Translation: (L)-Selenoaminoacids incorporation in proteins during translation	2.15	2.03
Heme metabolism	2.45	—
Mechanisms of cystic fibrosis transmembrane conductance regulator (CFTR) activation by S-nitrosoglutathione (normal and cystic fibrosis)	—	6.29
Regulation of degradation of deltaF508 CFTR in cystic fibrosis	—	3.94
CFTR folding and maturation (norm and cystic fibrosis)	—	2.96
Development: Glucocorticoid receptor signaling	—	2.49
Immune response: Antigen presentation by major histocompatibility complex (MHC) class I	—	2.36
Oxidative stress: Role of ASK1 under oxidative stress	—	2.19
Cholehepatic circulation of bile acids	—	2.10

^aRedundant pathways (e.g., same pathway in humans and rodents) have been removed.

for both species due to saturation of metabolism. The dose response for metabolism is complex because saturation occurs at different concentrations in the lung and liver (Sarangapani *et al.*, 2002). Model-predicted dose surrogates in the mouse were approximately four to eightfold higher than those in the rat for exposure concentrations greater than 1 ppm (Fig. 4).

DISCUSSION

Chloroprene is a rodent carcinogen with significant strain and species differences in sensitivity and organ specificity. In the rodent cancer bioassay performed by the National Toxicology Program, chloroprene produced a significant increase in tumors

across multiple sites and in both B6C3F1 mice and F344 rats (NTP, 1998). In the U.S. EPA's risk assessment for chloroprene (EPA, 2010a), lung tumors in the B6C3F1 mice were considered as one of the primary endpoints for carcinogenic risk because of the significant increase in incidence at the lowest exposure concentration (12.8 ppm). In contrast, only a slight, but not statistically significant, increase in lung tumors was observed at the highest exposure concentration (80 ppm) in female F344 rats.

The hypothesized mode of action for chloroprene involves the generation of reactive epoxide metabolites, leading to DNA damage and increased tumors (EPA, 2010b; Pagan, 2007). *In vitro* metabolism studies identified (1-chloroethenyl)oxirane as the primary metabolite, and it was sufficiently stable to be

TABLE 5
Pathways With Transcriptional BMD Values Consistent With Cross-Species BMD Values for Lung Tumor Responses

Pathway	Mouse					Rat			
	Total genes	5 days		15 days		5 days		15 days	
		Genes with BMD	Median BMD (ppm)	Genes with BMD	Median BMD (ppm)	Genes with BMD	Median BMD (ppm)	Genes with BMD	Median BMD (ppm)
Glutathione metabolism	33	21	5.85	18	5.85	11	146.61	9	83.70
Methionine-cysteine-glutamate metabolism	18	10	6.10	10	25.25	8	101.17	9	83.70
(L)-Alanine, (L)-cysteine, and (L)-methionine metabolism	24	10	9.27	9	49.65	11	134.60	8	96.67
Bile acid biosynthesis	21	8	9.99	11	12.04	10	148.00	3	189.40

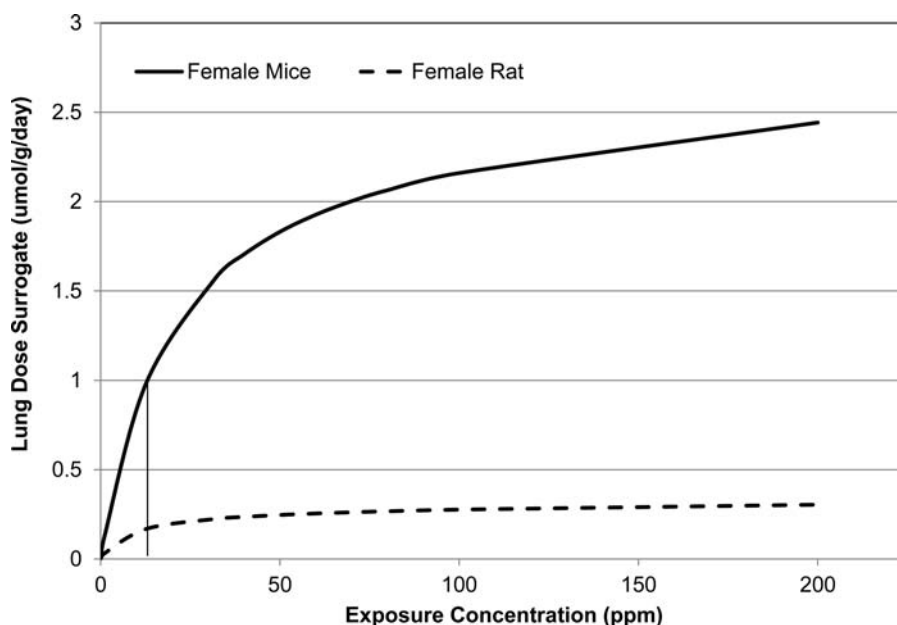


FIG. 4. The relationship between exposure concentration (ppm) and the dose surrogate of the total amount of chloroprene metabolized per unit mass of lung tissue per day ($\mu\text{mol/g lung/day}$) in female mice and rats. Female mice are represented by the solid black line and female rats are represented by the dashed black line. The dose surrogate in the female mouse lung at the lowest exposure concentration in the rodent cancer bioassay (12.8 ppm) is indicated by the vertical line.

structurally identified (Himmelstein *et al.*, 2001a). A second metabolite, (2-chloro-2-ethenyl)oxirane, was also identified, but it was much less stable *in vitro*, and its structure was inferred based on degradation products (Cottrell *et al.*, 2001). The primary isozyme responsible for chloroprene oxidation is Cyp2e1 (Cottrell *et al.*, 2001; Himmelstein *et al.*, 2001a). The (1-chloroethenyl)oxirane metabolite is a substrate for detoxification by epoxide hydrolase and glutathione *S*-transferase reactions (Himmelstein *et al.*, 2001a). The proposed breakdown products and secondary metabolism of (2-chloro-2-ethenyl)oxirane are much more complex with multiple secondary and tertiary metabolites that also include hydrolysis and conjugation with glutathione (Munter *et al.*, 2007). The specific carcinogenic metabolite(s) have not been conclusively identified; however, (1-chloroethenyl)oxirane

was mutagenic in *S. typhimurium* (Himmelstein *et al.*, 2001b) and shows reactivity with DNA *in vitro* (Munter *et al.*, 2002). Importantly, the rate of metabolic formation of the epoxide metabolites is highly species dependent. In lung microsomes, the rate of oxidative metabolism was much greater in mice than in other species (Himmelstein *et al.*, 2004b), and this has been proposed as the primary reason for the high incidence of lung tumors in female mice than in female rats (Himmelstein *et al.*, 2004a).

The term “toxicogenomics” was introduced into the literature over a decade ago with the expectation that combining the fields of toxicology and genomics would lead to the rapid identification and mechanistic understanding of chemical agents that result in adverse human health effects (Nuwaysir *et al.*, 1999). At the time, transcriptional analysis using microarrays was the primary

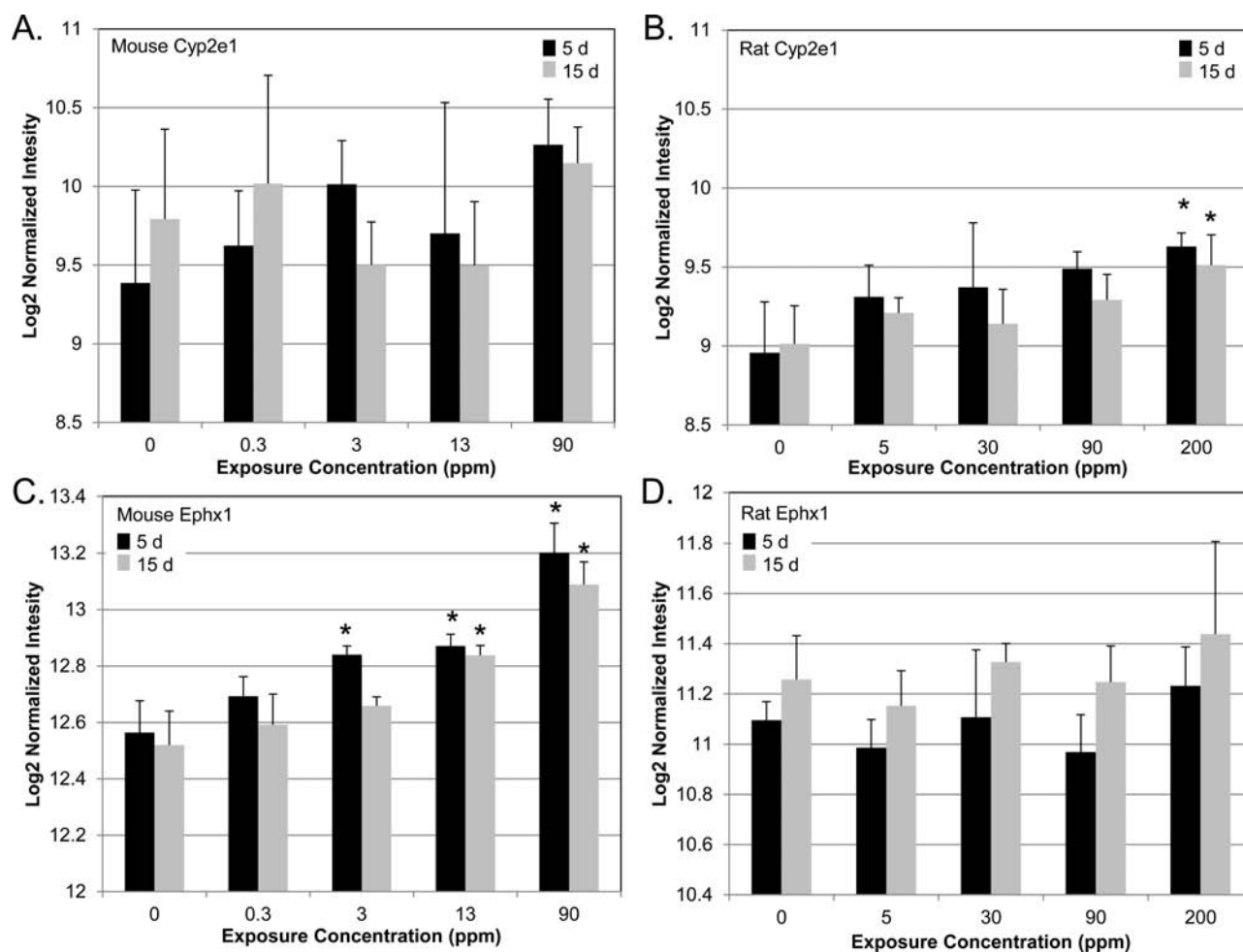


FIG. 5. Gene expression changes in key mouse and rat enzymes involved in chloroprene oxidation and hydroxylation. Expression changes in (A) mouse Cyp2e1, (B) rat Cyp2e1, (C) mouse Ephx1, and (D) rat Ephx1. Bars are mean \pm SD. Black and gray bars represent expression at the 5- and 15-day time points, respectively. * $p < 0.05$.

technology available. Although bioinformatic tools have continued to improve the interpretation of gene expression microarray data, the ability to identify the mechanism of action for a chemical based on gene expression microarray data is still a challenge. Multiple confounding issues contribute to this challenge including nonspecific effects of a chemical outside of the primary molecular mechanism, adaptive cellular responses to the chemical, temporal differences between phenotypic responses and gene expression changes, dissociation between transcriptional and protein-related changes, and downstream secondary and tertiary gene expression changes. In an attempt to filter out many of these confounding issues and help understand the potential mode of action of chloroprene in mouse lung tumors, we performed time-course and dose-response gene expression microarray analysis in the lung tumor-sensitive female B6C3F1 mice and compared the changes with those observed in the insensitive female F344 rats. Analysis of the gene expression changes was performed using both a traditional ANOVA followed by pathway enrichment analysis and pathway-based BMD analysis. In each

type of analysis, the pathways were examined to identify only those pathways with a dose response that was consistent with the cross-species differences in lung tumor incidence. In the enrichment analysis, a total of 18 pathways were enriched in the mouse lung at carcinogenic doses (≥ 12.8 ppm), and only one of these was also enriched in the rat lung at noncarcinogenic doses (≤ 80 ppm), leaving 17 pathways as potential candidates for involvement in the mode of action. In the pathway-based BMD analysis, a total of 11 pathways had median BMD values that were similar to the BMD value for chloroprene-induced lung tumors in the mouse. However, when these pathways were filtered to remove those with a median BMD value in the rat less than 80 ppm, only four pathways remained.

For the pathways identified using either the enrichment or BMD approaches, only two overlapped. These included glutathione metabolism and methionine-cysteine-glutamate metabolism. The ratios of the BMD values across species for these pathways were similar to the ratio of the BMD values for lung tumors. For the glutathione pathway, the cross-species

BMD ratio ranged from 14 to 25 depending on the time point (Table 5), whereas the cross-species BMD ratio for the tumor response was 28 (102 vs. 3.6 ppm). The transcriptional perturbation of the glutathione pathway is also consistent with the generation of reactive metabolites as a key event in the mode of action for chloroprene-induced lung tumors. A higher reactive metabolite burden in the mouse lung would translate into a greater dependence on reactivity with glutathione. Because glutathione is a tripeptide that is synthesized from L-cysteine, L-glutamic acid, and glycine (Pompella *et al.*, 2003), the transcriptional response in the methionine-cysteine-glutamate metabolism pathway is likely a secondary response to the glutathione biosynthesis. Further experimentation would be necessary to confirm the effect on glutathione-related gene expression by measuring and correlating *in vivo* glutathione status during *in vivo* chloroprene inhalation exposure.

Based on their consistency with the apical effect, the transcriptional changes in these pathways may be considered “bioindicators” of response (Preston, 2002). The cross-species BMD ratio for the glutathione pathway was more similar to the differences in the tumor response than the previously proposed target tissue dose surrogate of the total amount of chloroprene metabolized per unit mass of lung tissue per day (Himmelstein *et al.*, 2004a). The cross-species difference in the total amount of chloroprene metabolized per gram of lung tissue per day was on the order of four- to eightfold at concentrations greater than 1 ppm.

The current PBPK model was developed to account for total chloroprene oxidation in the basal state and does not include parameters for detoxification by epoxide hydrolase or induction of biotransformation enzymes resulting from treatment. For detoxification by epoxide hydrolase, the *in vitro* rate of metabolism has been measured in both mouse and rat microsomes (Himmelstein *et al.*, 2004b). In the mouse lung, the V_{\max}/K_m for chloroprene oxidation was 66.7 ml/h/mg protein, whereas hydrolysis was 2.1 ml/h/mg protein for an activation-to-detoxification ratio of 32:1. The corresponding V_{\max}/K_m values for the rat lung were 1.3 ml/h/mg protein for oxidation and hydrolysis, giving an activation-to-detoxification ratio of 1:1. The ratios suggest that the reactive metabolite burden would be as much as 30-fold higher in mouse lung than in the rat lung in the basal state. However, these *in vitro* measurements of metabolism may be confounded by dose-dependent increases in Cyp2e1 mRNA in the rat lung and microsomal epoxide hydrolase-1 (Ephx1) mRNA in the mouse lung observed in our studies (Fig. 5). It is not yet known whether the changes in Cyp2e1 and Ephx1 mRNAs are translated into increased enzyme activity, but the ultimate result would be a narrowing of the cross-species differences in the activation-to-detoxification ratios.

The closer association of the glutathione transcriptional response with tumors may be a reflection of either greater cross-species differences in the amount of reactive metabolites formed in the target cell type (likely Clara cells richer in cytochrome P450 enzymes) as opposed to normalizing across

the whole lung or the potential cross-species differences in detoxification and antioxidant responses, which are not included in the current PBPK model. Interestingly, the cross-species differences in the pathway BMD values for the most sensitive pathways (i.e., those with the lowest median BMD values) were more closely aligned with the difference in the total amount of chloroprene metabolized per gram of lung tissue. Pathway BMD values for the most sensitive pathways occurred at similar target tissue dose surrogates in both species (~0.2 $\mu\text{mol/g/day}$). Notably, the dose surrogate in the female mouse lung at the lowest exposure concentration in the rodent cancer bioassay (12.8 ppm) was approximately 1 $\mu\text{mol/g/day}$ and was never reached in the female rat lung at any exposure concentration.

In conclusion, the cross-species transcriptomic results are consistent with the generation of reactive metabolites as a key event in the mode of action for chloroprene-induced lung tumors. Further, based on cross-species BMD ratios, the pathway-based transcriptional responses appear to be an improved surrogate for lung tumor risk than previous surrogates based solely on chloroprene oxidative metabolism and pharmacokinetics. Application of the cross-species transcriptomic studies to organotypic *in vitro* models of human lung cells may help assess the relative risks of chloroprene exposure to humans. Conserved responses may differ in magnitude between species, but if the differences are consistent, then appropriate adjustment factors can be derived for cross-species inferences (NRC, 2007).

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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